Statement on Dairy-20 (*Clostridium butyricum*) Taxonomy Change to *Clostridium beijerinckii* ASCUSDY20 on the Basis of Genome Analysis

(Note original deposit to NRRL B-67248 was identified as *Clostridium butyricum.* This document supports the name change to *Clostridium beijerinckii* ASCUSDY20)

Dairy-20 (*Clostridium butyricum*) was identified and isolated to axenicity from a healthy, mid-lactation Holstein cow rumen sample that was obtained via cannula in 2015. It is an anaerobic, catalase, and oxidase negative bacterium that readily sporulates. The microorganism is gram-positive and forms long chains of small cocci when cultured in liquid medium. Initial phenotypic analysis using UPLC demonstrated that Dairy-20 *Clostridium butyricum* produces butyric acid but not butanol. Taken alongside its carbon use according to the API 20E carbon panel (BioMérieux, Marcy-l'Étoile, France), its phenotype was consistent with that typical of *Clostridium butyricum,* due to its production of butyric acid (which is produced by both species) but not butanol, which at the time was considered to be a distinguishing characteristic of *Clostridium beijerinckii* (Keis *et al.*, 2001; Crabbendam *et al.*, 1985; Heyndrickx *et al.*, 1991; Zigova *et al.*, 1999).

To confirm the taxonomic assignment, 16S rRNA gene analysis was used (Wilson *et al.*, 1990). The 16S sequence was compared to NCBI databases to establish the identity of the strain. This analysis showed greater than 99% identity to the 16S sequences of *Clostridium saccharoperbutylacetonicum, Clostridium butyricum*, and *Clostridium beijerinckii.* Based on the 16S marker, all three species fall within the minimum 98.7% sequence identity threshold that is typically used to define a species (Yarza *et al.*, 2014). At the time (2015), this evidence was considered sufficient to support the phenotypic data assuming that the absence of butanol production excluded classification as *Clostridium beijerinckii*. Thus, Dairy-20 was designated as a strain of *Clostridium butyricum*.

Since the original identification the whole genome of Dairy-20 *Clostridium butyricum* has been sequenced, and further genetic comparisons have been carried out. Average nucleotide identity (ANI) is a recent method that quantifies the nucleotide-similarity between the coding regions of two genomes (Jain et al., 2018). MUMmer was used to generate the alignments for ANI: this software is efficient at aligning highly similar sequences and is more stringent than most other aligners such as BLAST (Kurtz *et al.*, 2004). ANI comparison of Dairy-20 *Clostridium butyricum* with a strain *Clostridium beijerinckii* provided the highest identity of 98%, with 89% coverage. The identity to *Clostridium butyricum* was only 84% with 8% coverage. In addition, comparison of the sequences of four housekeeping genes ribonuclease P RNA (rnpB), ATP synthase alpha subunit (atpA), RNA polymerase alpha subunit (rpoA), and phenylalanyl-tRNA

synthetase (pheS) was performed. All four housekeeping genes of Dairy-20 *Clostridium butyricum* were at least 99% identical to those in *Clostridium beijerinckii*, while matches to *Clostridium butyricum* were only possible for rpoA and atpA, with 92.3% and 83.7% identity respectively. The genome size of Dairy-20 *Clostridium butyricum* (5.98 Mbp) is also consistent with classification as *Clostridium beijerinckii* (typical strain genome size 6.0 Mbp [NCBI, 2019a]) and not *Clostridium butyricum* (typical strain genome size 4.64 Mbp [NCBI, 2019b]).

In light of the new and conclusive genetic evidence, the classification of the Dairy-20 *Clostridium butyricum* strain has been re-evaluated. The characterization data available indicate that Dairy-20 is a strain of *Clostridium beijerinckii* which is phenotypically atypical in that it does not produce butanol. *Clostridium beijerinckii* strains are largely selected for commercial butanol production, but strains which do not produce significant butanol are known (Alam *et al*., 1988). *Clostridium beijerinckii* has been identified as a normal member of the rumen microbiome in multiple studies (Ho *et al*., 2011; Hoang *et al*., 2018; Marichamy and Mattiasson 2005; Sankar *et al*., 2003; Seshadri *et al*. 2018). There are no reports of pathogenicity, toxigenicity and literature searches have not identified any other safety concerns relevant to this organism. We are therefore proposing to reclassify Dairy 20 as *Clostridium beijerinckii* ASCUSDY20, and this name change has no effect on function, utility or safety.

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BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSE OF PATENT PROCEDURES

INTERNATIONAL FORM

TO
Patent Dept.
Ascus Biosciences
6450 Lusk Blvd. E102/209
San Diego, CA 92121

NAME AND ADDRESS OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: *Clostridium butyricum* *Ascusb_3138*	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NRRL B-67248

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

☐ [1]a scientific description

☐ [1]a proposed taxonomic designation

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on
April 25, 2016 (date of the original deposit)[2]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I. above, was received by this International Depositary Authority on _____
(date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received
by it on _____ (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name:	Agricultural Research Culture Collection (NRRL) International Depositary Authority	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):
Address:	1815 N. University Street Peoria, Illinois 61604 U.S.A.	Date: April 27, 2016

[1] Mark with a cross the applicable box.
[2] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Objectives

The objective of this work was to assess the carbohydrate degradative capabilities and metabolite production of *Clostridium beijerinckii* ASCUSDY20 through *in vitro* assays.

Methods

To determine carbon source utilization of Arabinose, Ribose, Mannitol, Sorbitol, Gluconate, Xylose, Cellobiose, and Starch, ▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮ (b) (4)

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▮▮▮▮▮▮▮▮▮▮▮▮. Anaerobic solutions were sparged ▮▮▮▮▮▮▮▮ (b) (4) Prior to autoclaving the media was pH adjusted to (b) (4) ▮▮▮ of a single *C. beijerinckii* ASCUSDY20 colony that was inoculated into 10 mL of RAMM salts buffer was added to their respective well. *C. beijerinckii* ASCUSDY20 was incubated at 37°C. OD600 readings were taken prior to incubation. *C. beijerinckii* ASCUSDY20 was incubated for 165.67 hours. After incubation OD600 was measure again to determine organism growth. ▮▮▮▮▮▮▮▮ (b) (4)

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▮▮▮▮▮▮ Experimental Information can be found in Ascus Biosciences Notebook #13 Norman Pitt.

To determine carbon source utilization of Fructose, Dextrose, Lactose, Maltose, Sucrose, Cane Molasses, Beet Molasses, Lactate, Succinate, and Glycerol, the following growth media was prepared per 500 mL: ▮▮▮▮▮▮ (b) (4)

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▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮ After autoclaving the media was moved to an anaerobic environment and ▮▮▮▮▮▮▮▮ (b) (4)

▮▮▮▮▮▮▮▮▮▮ Each of the following carbon sources were prepared at ▮▮▮ (b) (4) ▮▮▮▮▮▮▮▮▮▮ dextrose, lactose, maltose, sucrose, cane molasses, beet molasses, fructose, glycerol. Acetic acid was added at ▮▮▮▮▮▮ (b) (4) ▮▮▮▮▮▮. Lactic Acid and succinic acid were at a concentration of ▮▮▮▮ (b) (4) ▮▮▮▮▮▮

▮▮▮▮ To each well of an anaerobic 96 well plate 0.8 mL of the above media was added. For each carbon source 0.08 mL were added to their respective well. Then 8 μL of preculture was added to the necessary wells. The samples were then incubated at 37°C for 48 hours. At 24 hours and 48 hours post inoculation 80 μL of culture was removed and the OD600 was measured. Experimental Information can be found on Google Drive at Ascus Biosciences/Lab/Process Development/Strains/Dairy (DY)/DY20/Carbon_source_panel_DY20.xlsx.

Metabolite production of *C. beijerinckii* ASCUSDY20 fermentation run 1801.32 U20 was measured at 48 hours using an Agilent 1260 series with RI detector operated at (b) (4). The column used was a Bio-Rad Aminex HPX-87H #1250140 with Bio-Rad Cation H+ guard #1250129 operated at (b) (4). The mobile phase was ⟨(b) (4)⟩. Pure standards were used at varying concentrations to generate a standard curve.

Results

C. beijerinckii ASCUSDY20 was assessed for growth on a variety of carbon sources. The carbon source utilization data is shown in Table 1.

Table 1: Growth of *Clostridium beijerinckii* ASCUSDY20 on Different Carbon Sources

Carbon Source	Growth	Carbon Source	Growth
Arabinose	+	Gluconate	-
Ribose	-	Xylose	+
Mannitol	+	Cellobiose	+
Sorbitol	+	Starch	+
Fructose	+	Dextrose	+
Lactose	+	Maltose	+
Sucrose	+	Cane Molasses	+
Beet Molasses	+	Acetate-Dextrose	+
Lactate	-	Succinate	-
Glycerol	-		

The metabolite production of *C. beijerinckii* ASCUSDY20 at 48 hours of growth on a complex media with glucose as the major carbon source was assessed in Table 2.

Table 2: Metabolite production of *Clostridium beijerinckii* ASCUSDY20 grown complex media with Glucose

Metabolite	Production
Pyruvic	-
Succinic	-
Lactic	-
Glycerol	-
Acetic	+
Propionic	-
Butyric	+
Ethanol	+
1-Butanol	+

Conclusions

C. beijerinckii ASCUSDY20 is able to utilize multiple carbon sources for growth including monosaccharides, disaccharides, and polysaccharides. *C. beijerinckii* ASCUSDY20 was found to produce acetic acid, butyric acid, ethanol, and 1-Butanol as metabolites when grown on a complex media with glucose as the main carbon source.

Signed _____ Date: 14 Jan 2020

Objectives

The objective of this work was to determine the identity of *Clostridium beijerinckii* ASCUSDY20 using genomic methods.

Methods

For 16S sequence analysis, the 16S gene was amplified from *C. beijerinckii* ASCUSDY20Dairy-20 using the 27F/534R primers and sequenced using an Illumina Miseq (Stackebrandt and Goodfellow 1991; Muyzer, de Waal, and Uitterlinden 1993; LANE and J 1991). The resulting sequence was quality trimmed and compared to NCBI databases (excluding "uncultured" and environmental samples) to establish the identity of the strain. The NCBI databases were queried on November 14, 2019.

For whole genome average nucleotide identity (ANI) analysis, genomic DNA was isolated via bead-based lysis using the MoBio PowerViral DNA kit (Carlsbad, CA). Sequencing libraries were prepared using the Nextera XT kit (Illumina, San Diego, CA), and the resulting libraries were paired-end sequenced (2x300bp) on an Illumina Miseq. The draft genome was assembled using SPAdes [version 3.6.2] (Bankevich et al., 2012). MUMmer was used to generate the alignments for whole genome average nucleotide identity (ANI) (Kurtz et al., 2004).

Results

C. beijerinckii ASCUSDY20 aligned with multiple isolates from *Clostridium saccharoperbutylacetonicum*, *Clostridium butyricum*, *Clostridium beijerinckii*, and *Clostridium diolis* above the 98.7% threshold used to define a species (Yarza et al. 2014). The best match for each species is listed in Table 1 below. The full list of hits and alignments can be seen in a separate file described in the documentation section.

Table 1: 16S analysis of *C. beijerinckii* ASCUSDY20

Genus species (accession #)	Percent Match	Percent Coverage
Clostridium saccharoperbutylacetonicum N14HMT (NR_102516.1)	100%	100%
Clostridium beijerinckii NCIMB 14988 (CP010086.2)	99.37%	100%
Clostridium butyricum subsp.convexa (AB647330.1)	98.95%	100%
Clostridium diolis DSM 15410 (CP043998.1)	98.74%	100%

Whole genome average nucleotide identity (ANI) was used to confirm the 16S identification. Genomes from multiple strains for each genus/species match from the 16S analysis were assessed using ANI (% identity and coverage). As shown in Table 2, the *C. beijerinckii* ASCUSDY20 genome most closely matched *Clostridium beijerinckii.*

Table 2: Whole Genome ANI analysis of *C. beijerinckii* ASCUSDY20

Genus species (GenBank accesion #)	ANI (%)	Coverage (%)
Clostridium beijerinckii SA 1 (GCA_000767745)	98.4	88.9
Clostridium_beijerinckii NCIMB 8052 (GCA_000016965)	98.4	88.8
Clostridium diolis DSM15410 (GCA_008580445)	97.7	84.8
Clostridium diolis WST (GCA_003015255)	97.7	83.5
Clostridium diolis NJP7 (GCA_002176895)	97.6	85.7
Clostridium beijerinckii NRRL B598 (GCA_000506785)	97.6	81.5
Clostridium beijerinckii BASB3I124 (GCA_002003345)	97.4	83.6
Clostridium beijerinckii NCIMB 14988 (GCA_000833105)	97.2	78.5
Clostridium beijerinckii DSM 6423 (GCA_900010805)	95.8	77.9
Clostridium saccharoperbutylacetonicum N1504 (GCA_002003305)	85.9	20.9
Clostridium saccharoperbutylacetonicum N14HMT (GCA_000340885)	85.8	20.0
Clostridium butyricum CDC51208 (GCA_001886875)	84.8	8.41
Clostridium butyricum JKY6D1 (GCA_001465175)	84.4	8.32
Clostridium butyricum KNUL09 (GCA_001456065)	84.4	8.24

Clostridium butyricum TOA (GCA_001646605)	84.3	8.28

Conclusions

C. beijerinckii ASCUSDY20 most closely matched the whole genome assemblies of two *Clostridium beijerinckii* strains by ANI. There are strains of *Clostridium diolis* that provide strong matches as well. The genomic data in this Appendix should be used along with the phenotypic data from Appendix 2 to confirm the identity as *C. beijerinckii* ASCUSDY20.

Documentation
The full list of 16S hits and alignments can be found on the Ascus drive under:

/AscusBiosciences/James_Analysis/Regulatory/DY20_Regulatory_Analysis/DY20_GRAS_Docs
/191114_Dairy-20_Alignment.txt

The details of the ANI analysis can be found on the Ascus drive under:

/AscusBiosciences/James_Analysis/Regulatory/DY20_Regulatory_Analysis/DY20_GRAS_Docs
/DY20_GRAS_ANI/

Signed: _____ Date: 11/19/19

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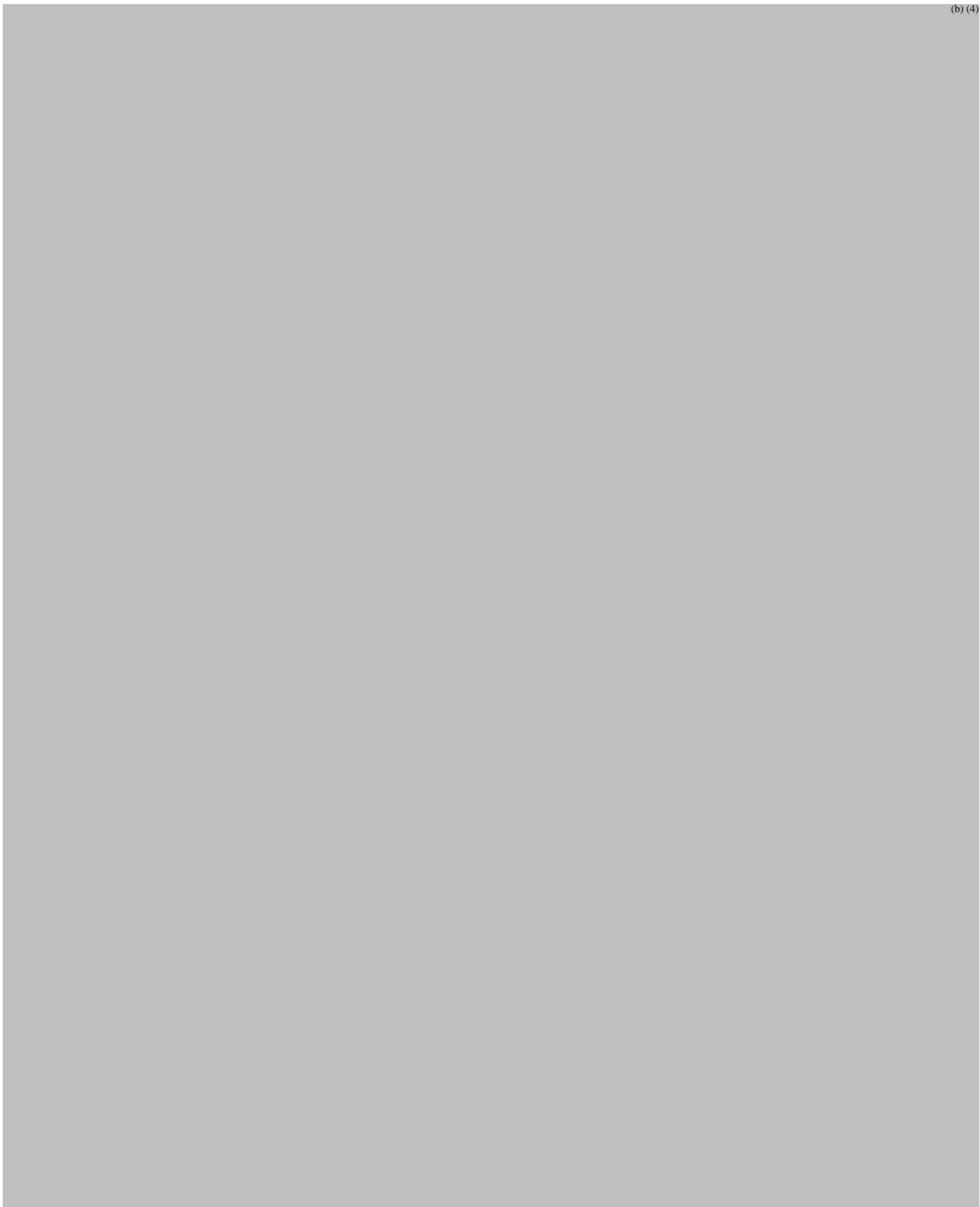
RID: WY9MYDCS014
Job Title:Nucleotide Sequence
Program: BLASTN
Database: nt Nucleotide collection (nt)
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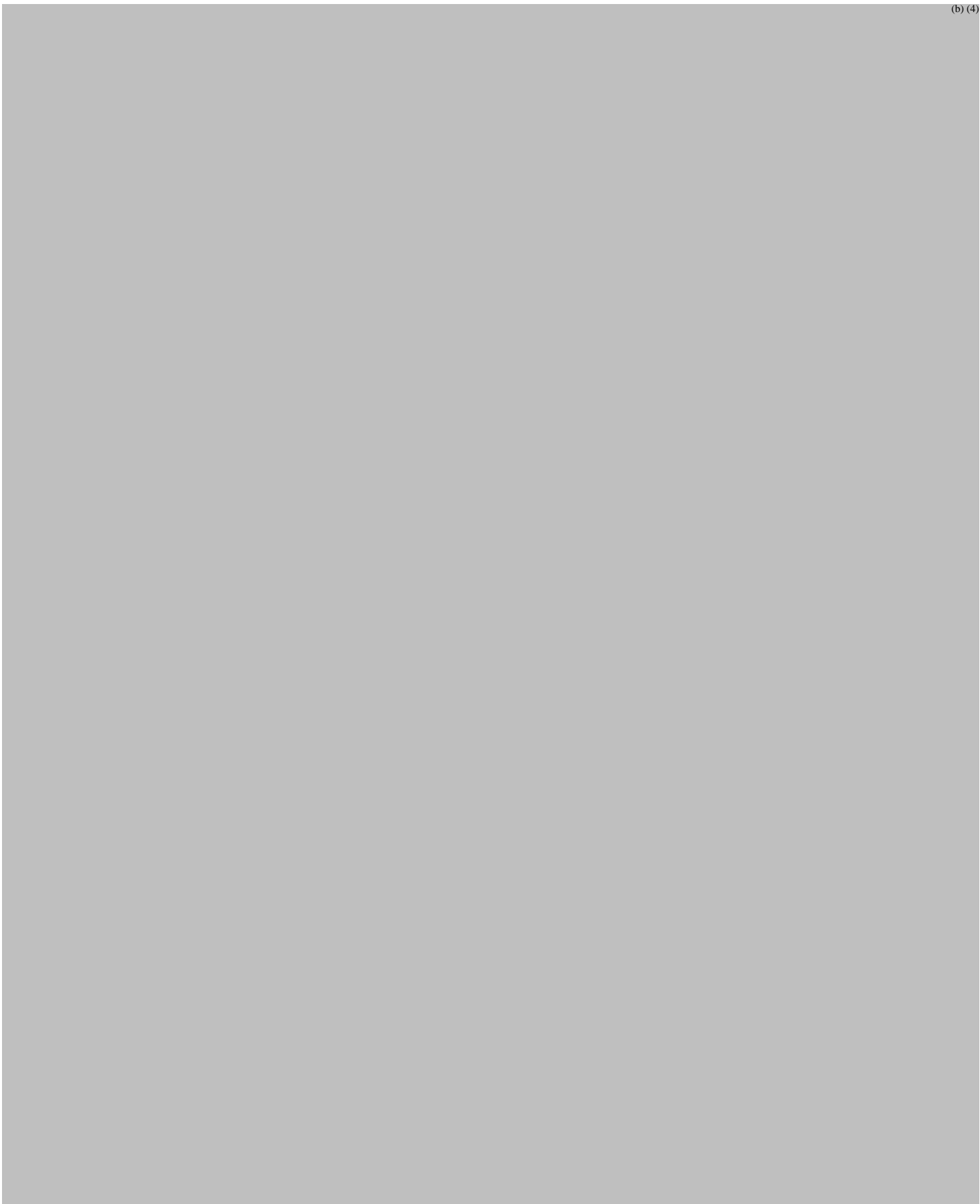Sequences producing significant alignments:

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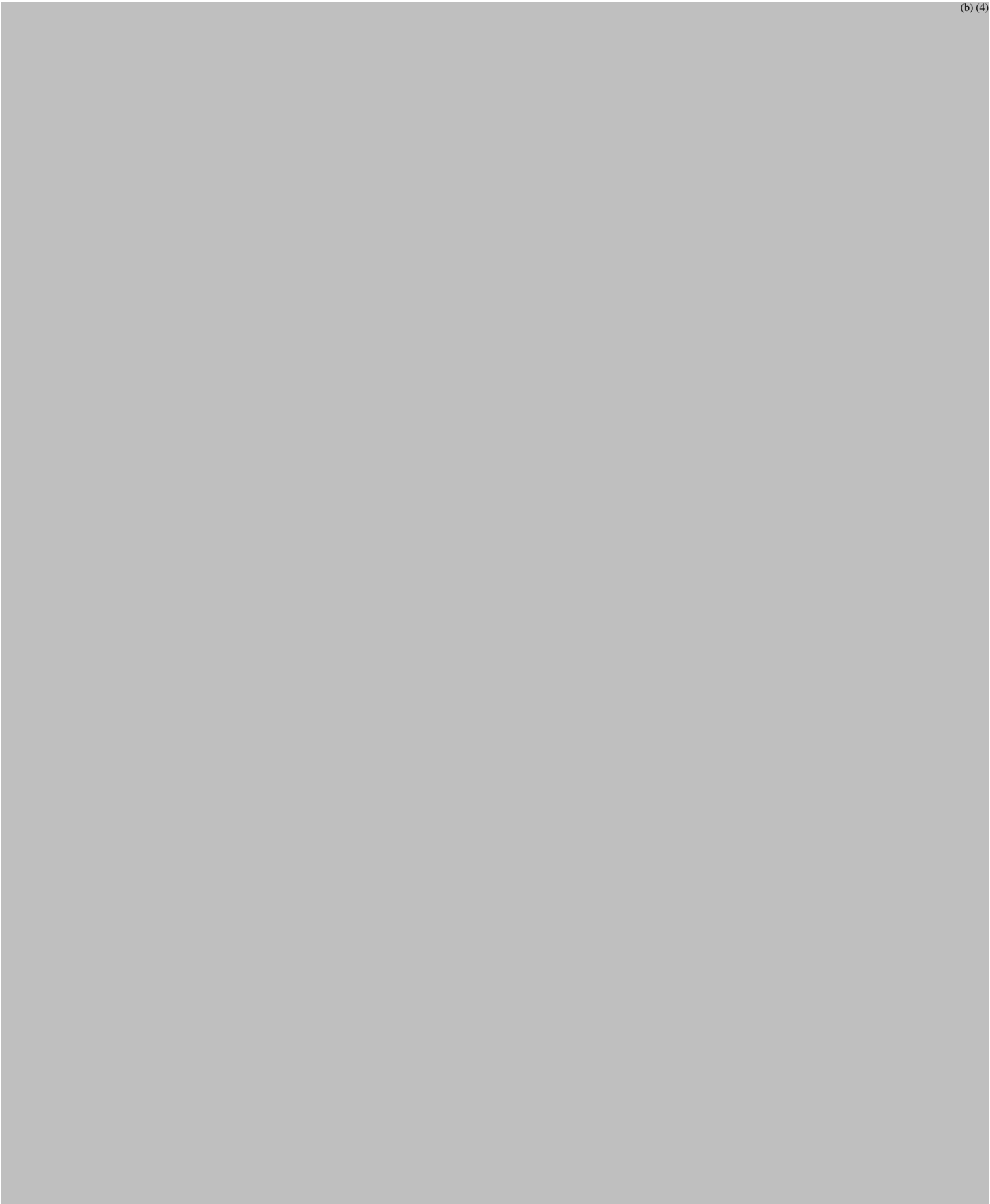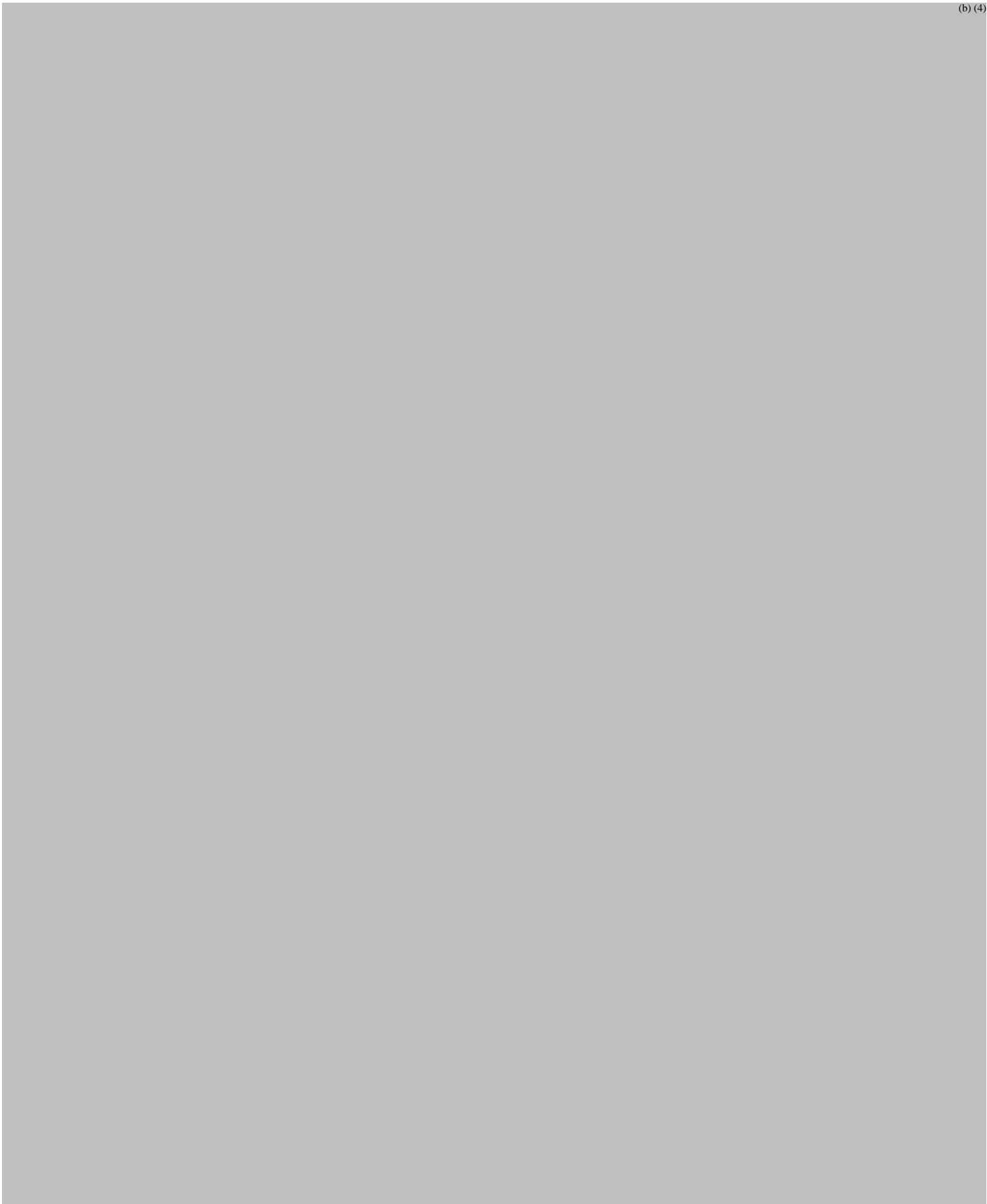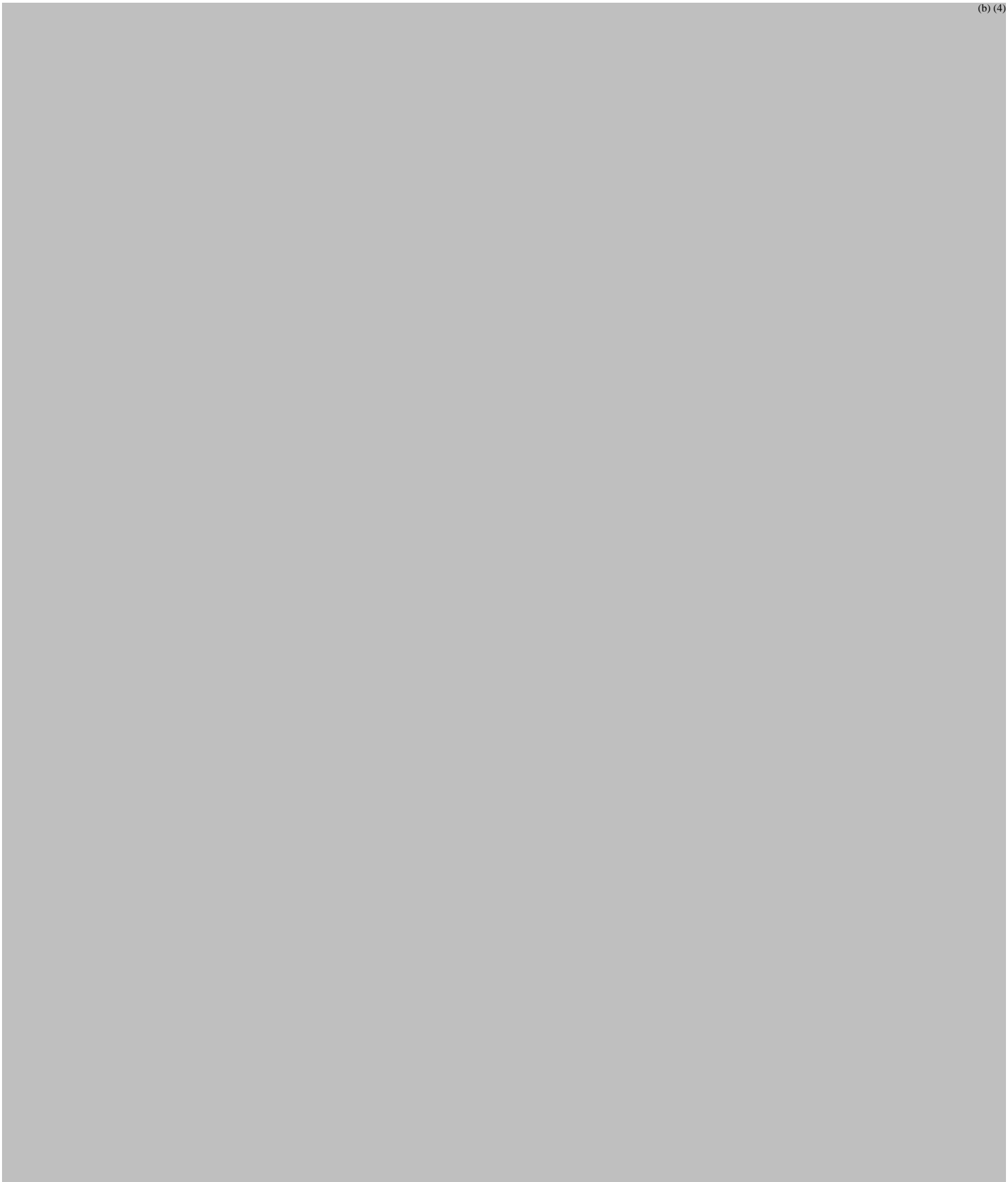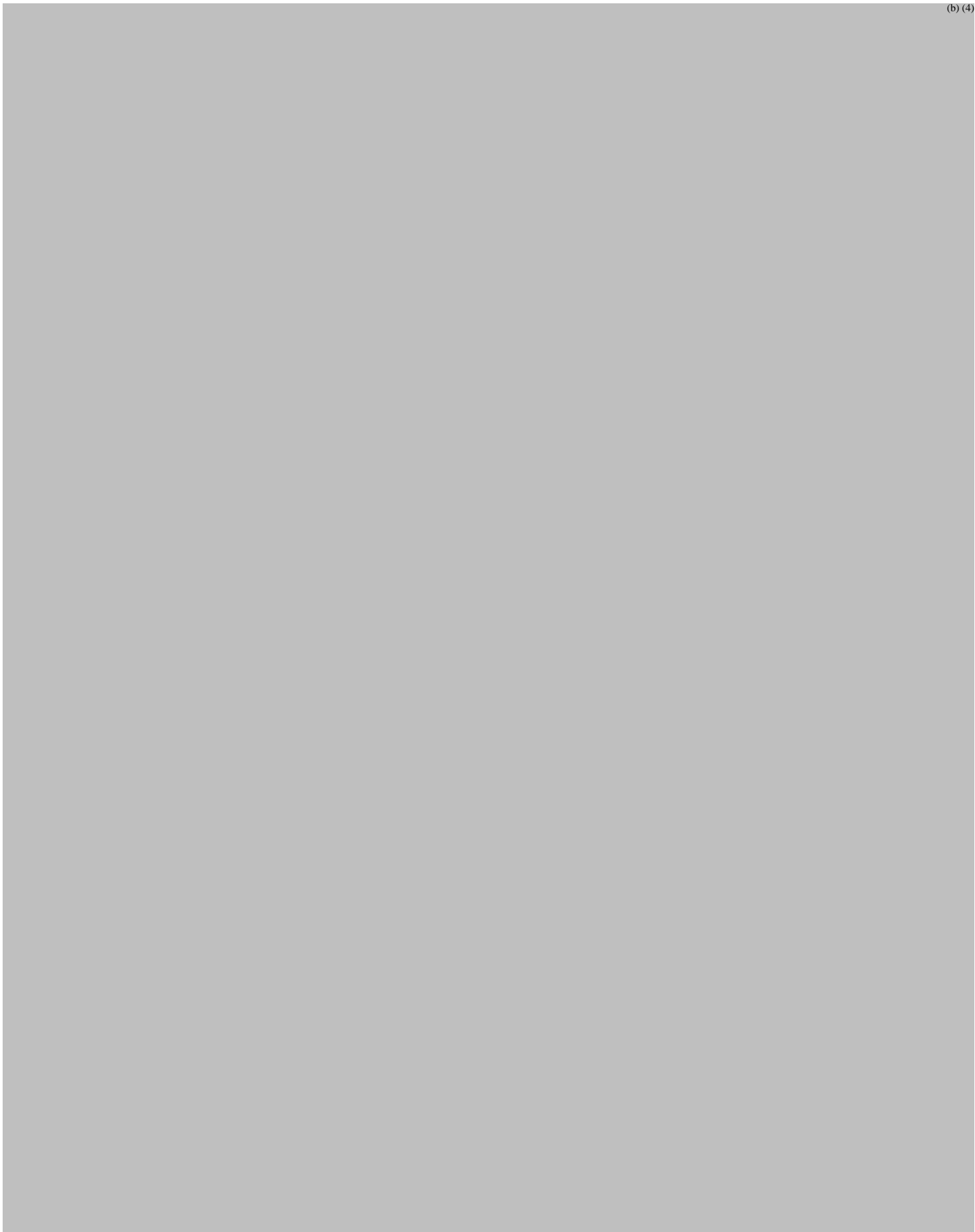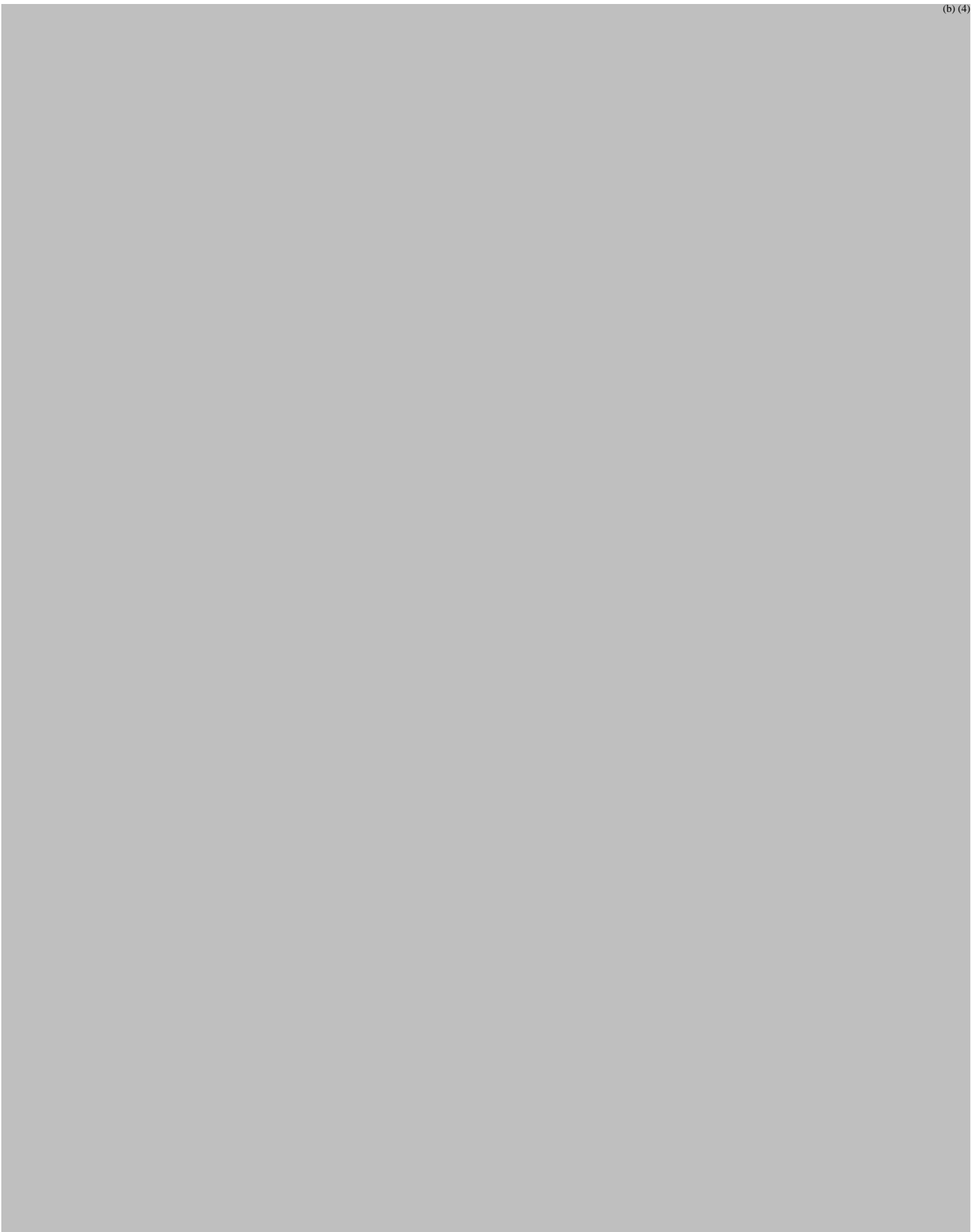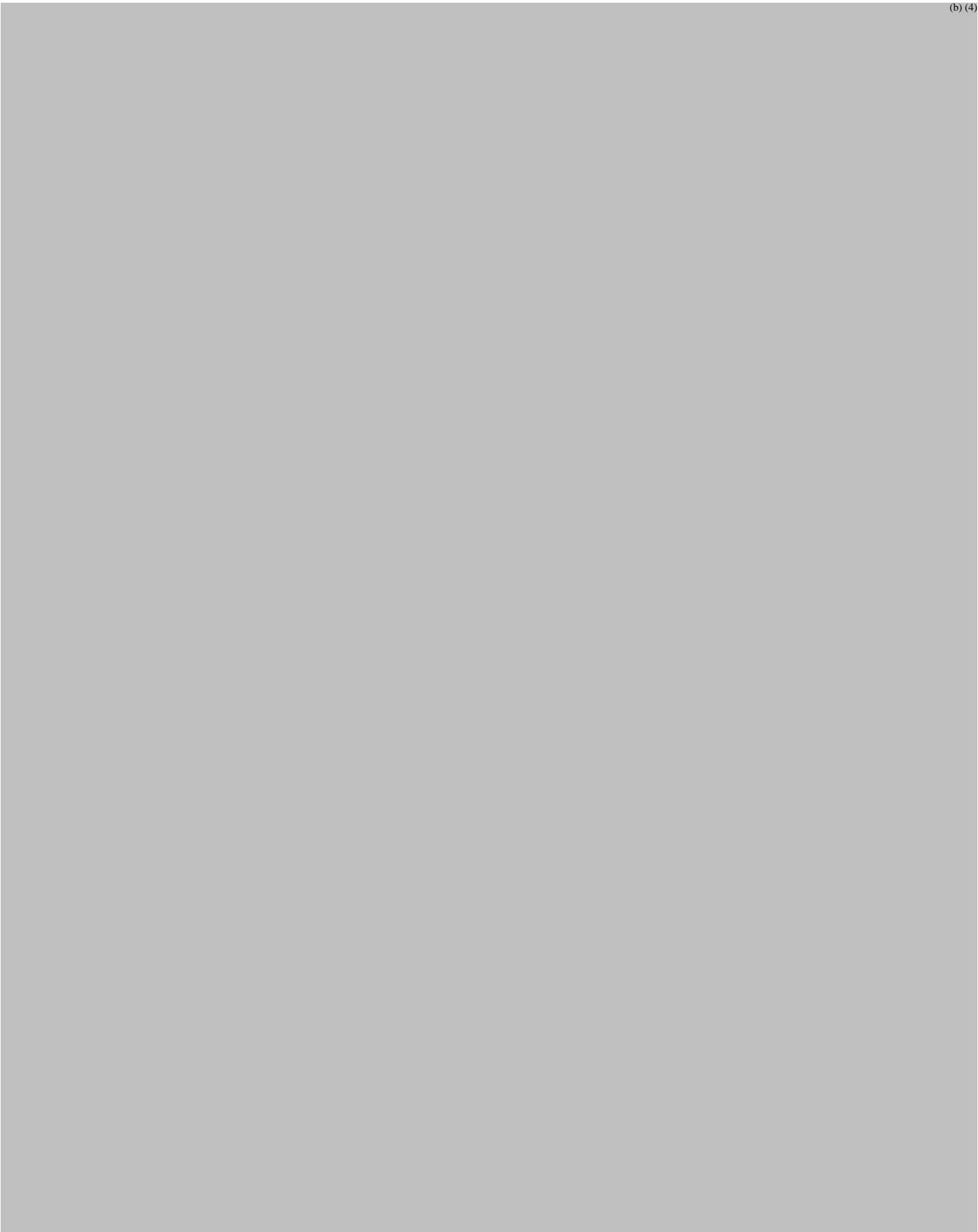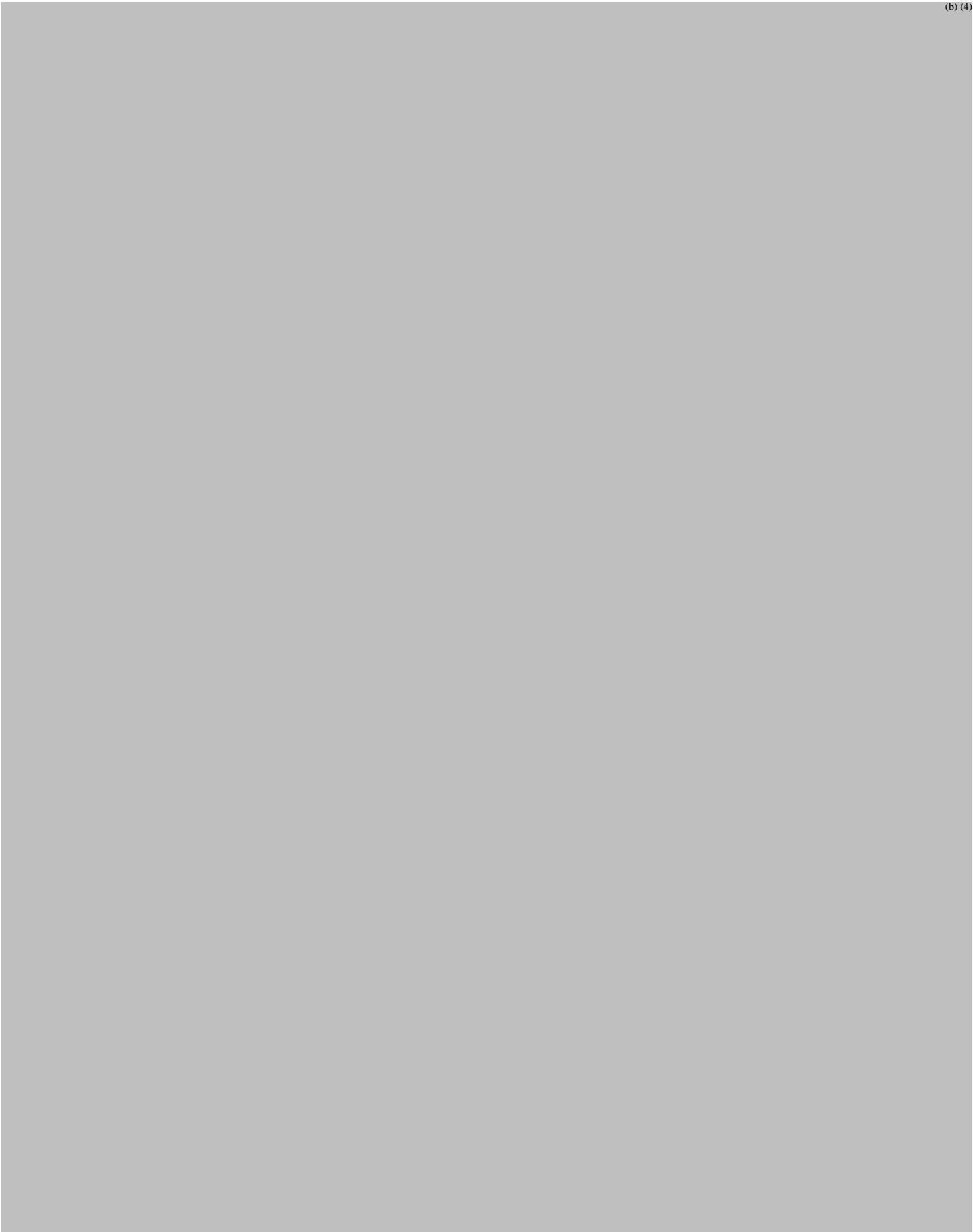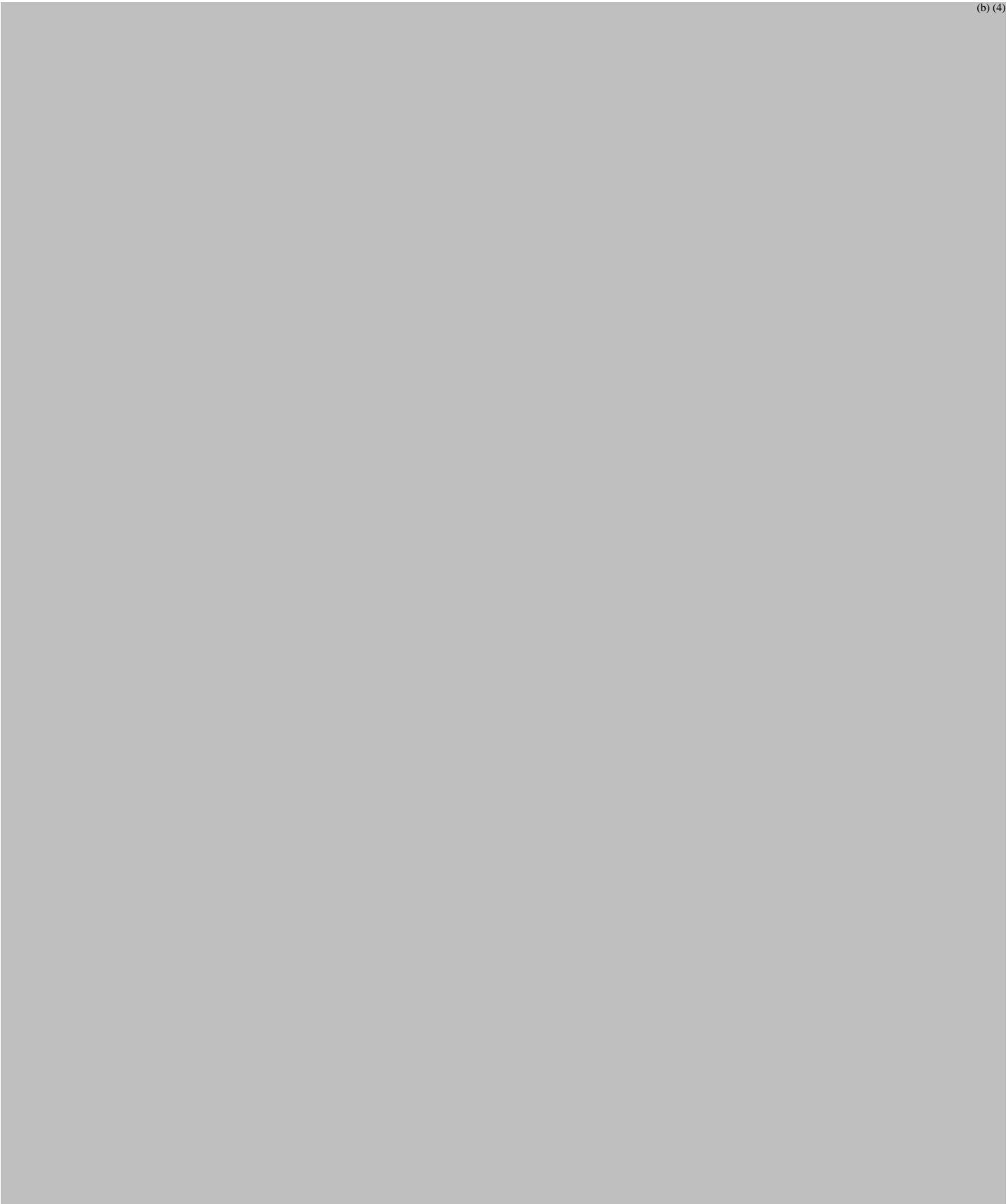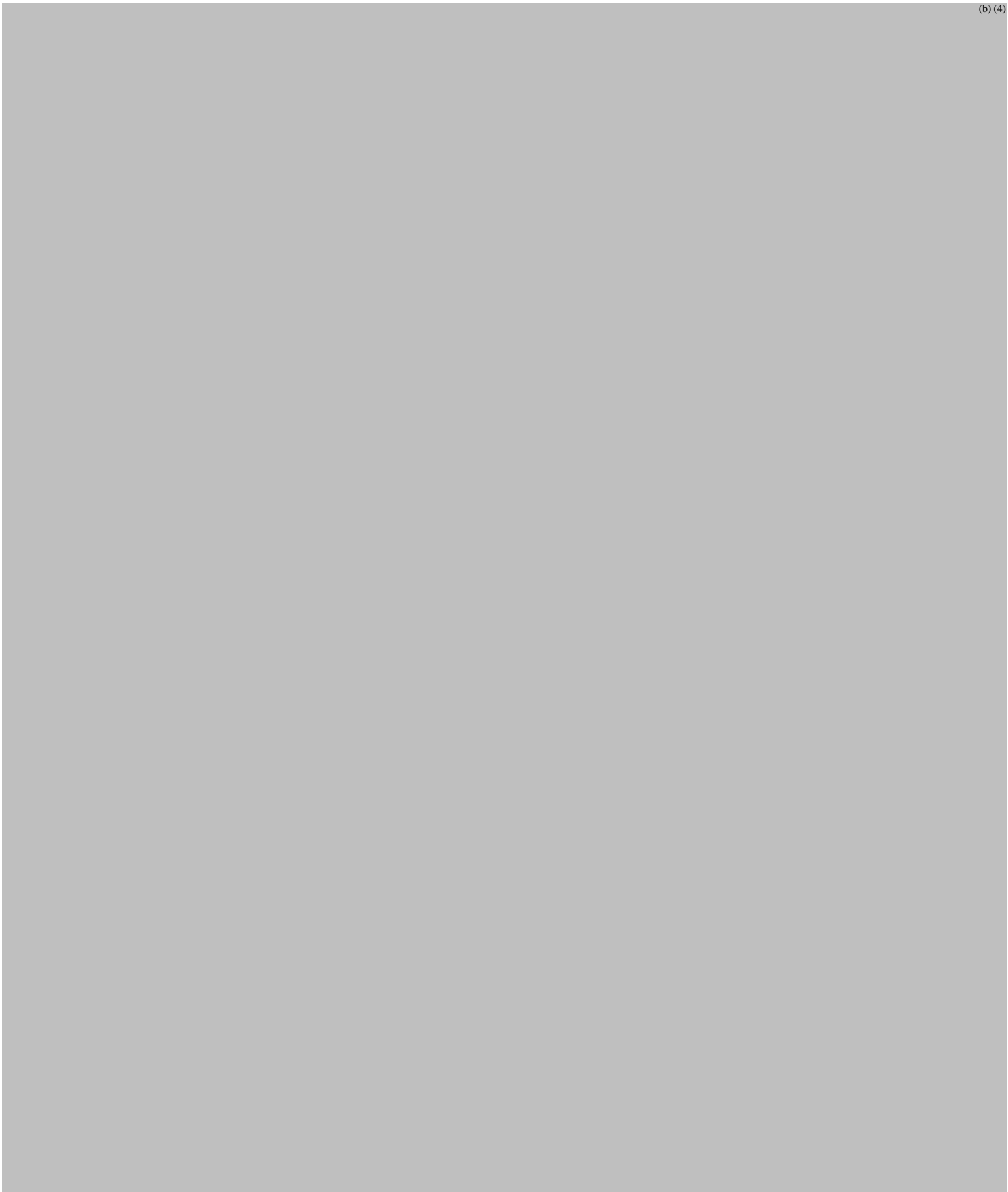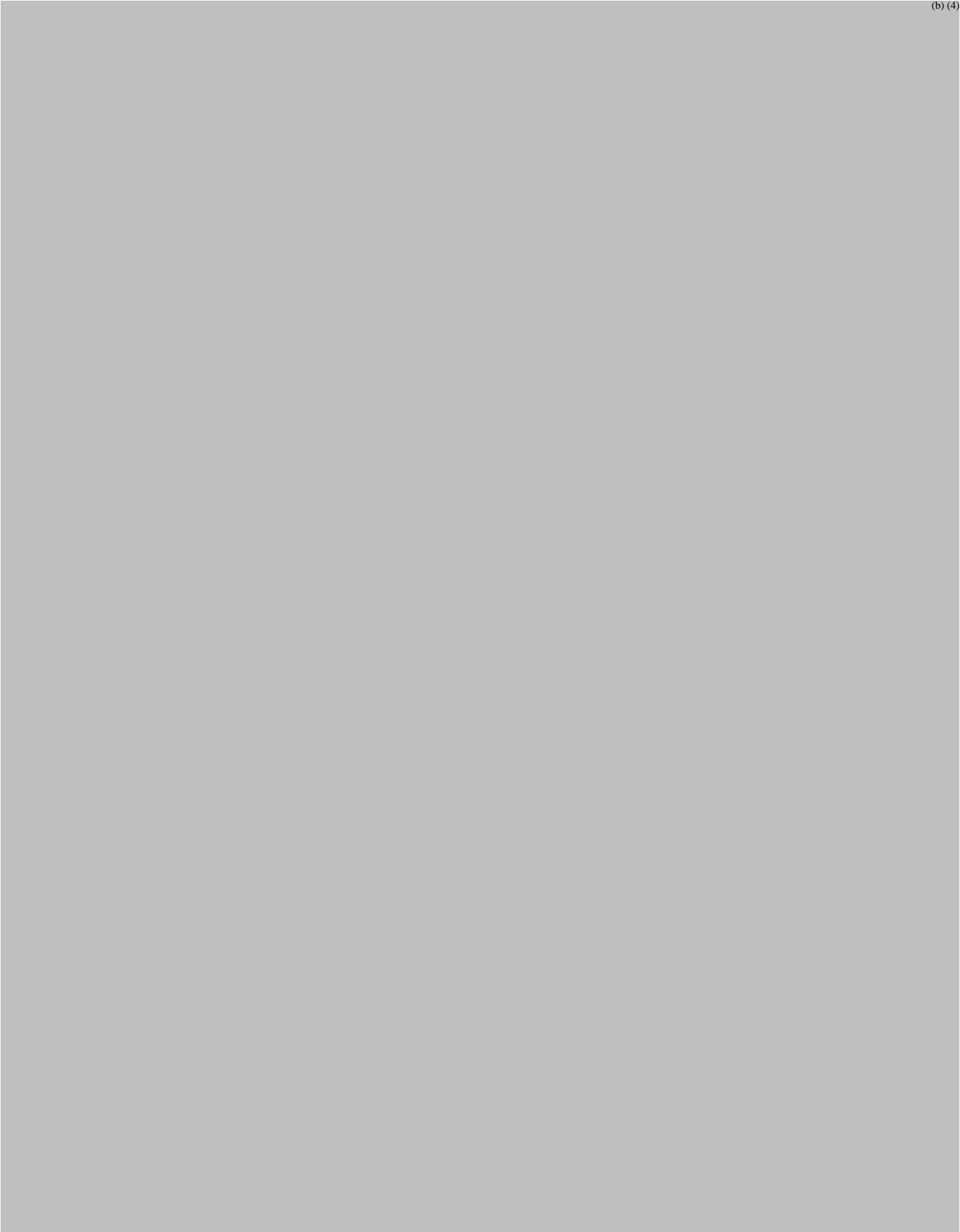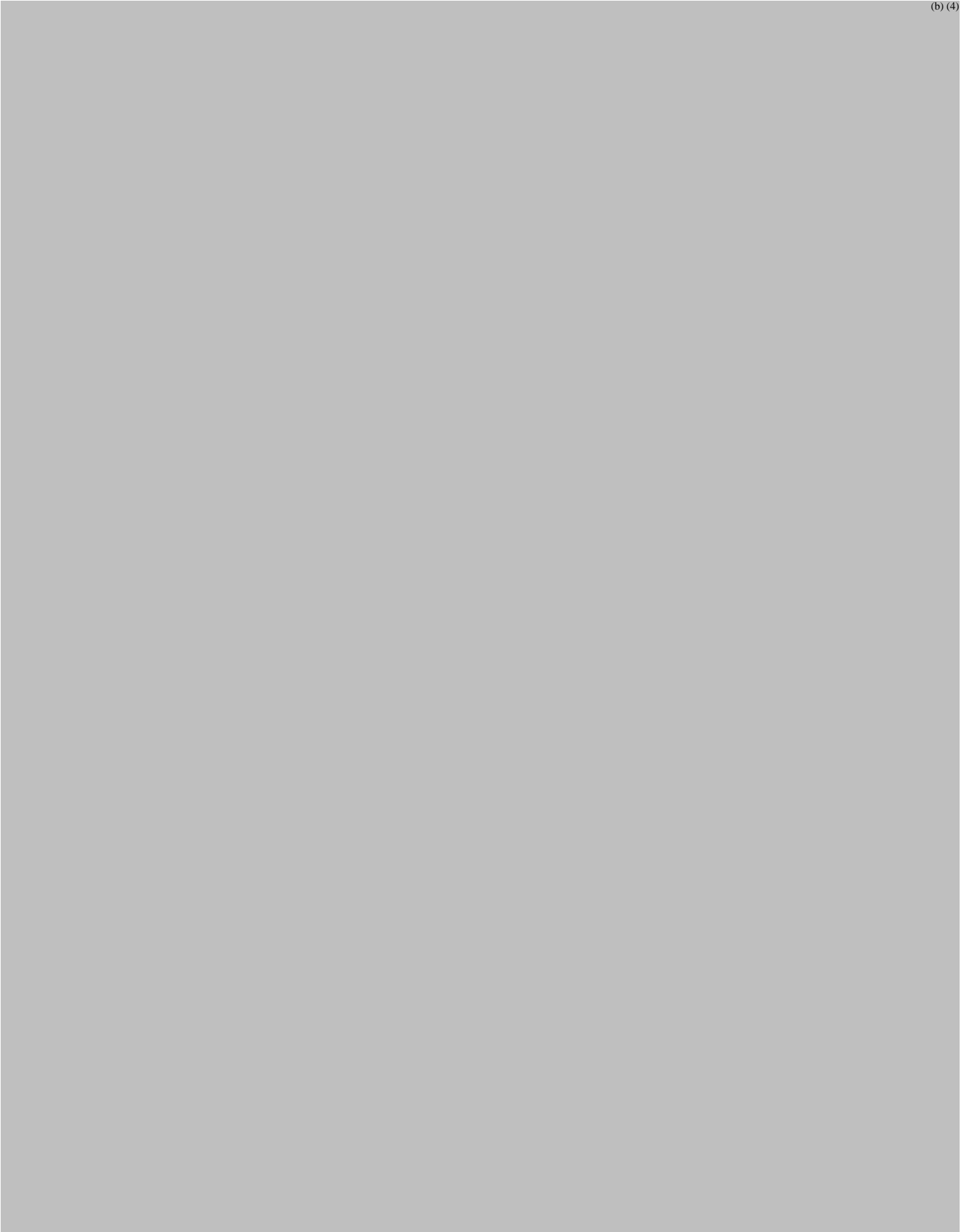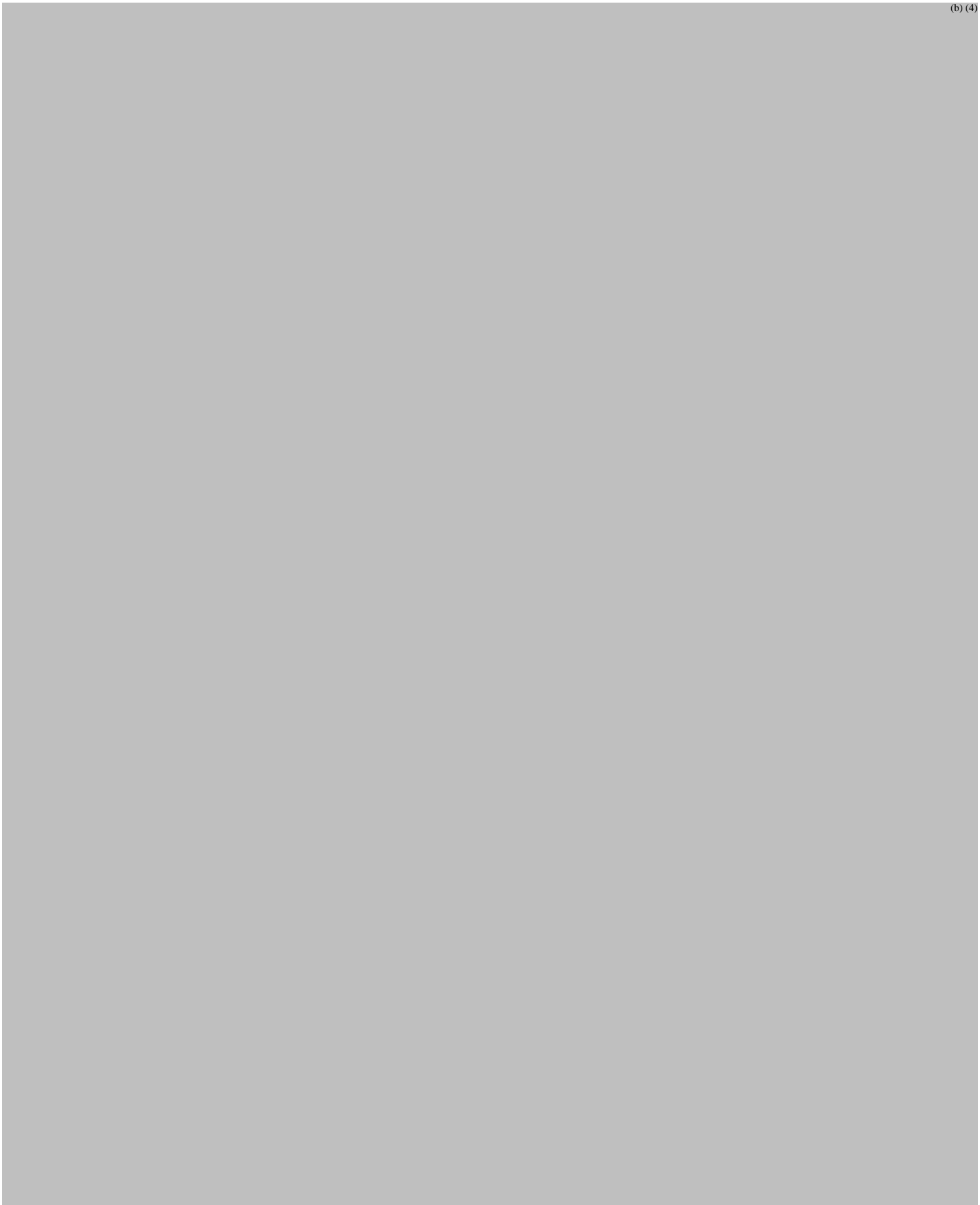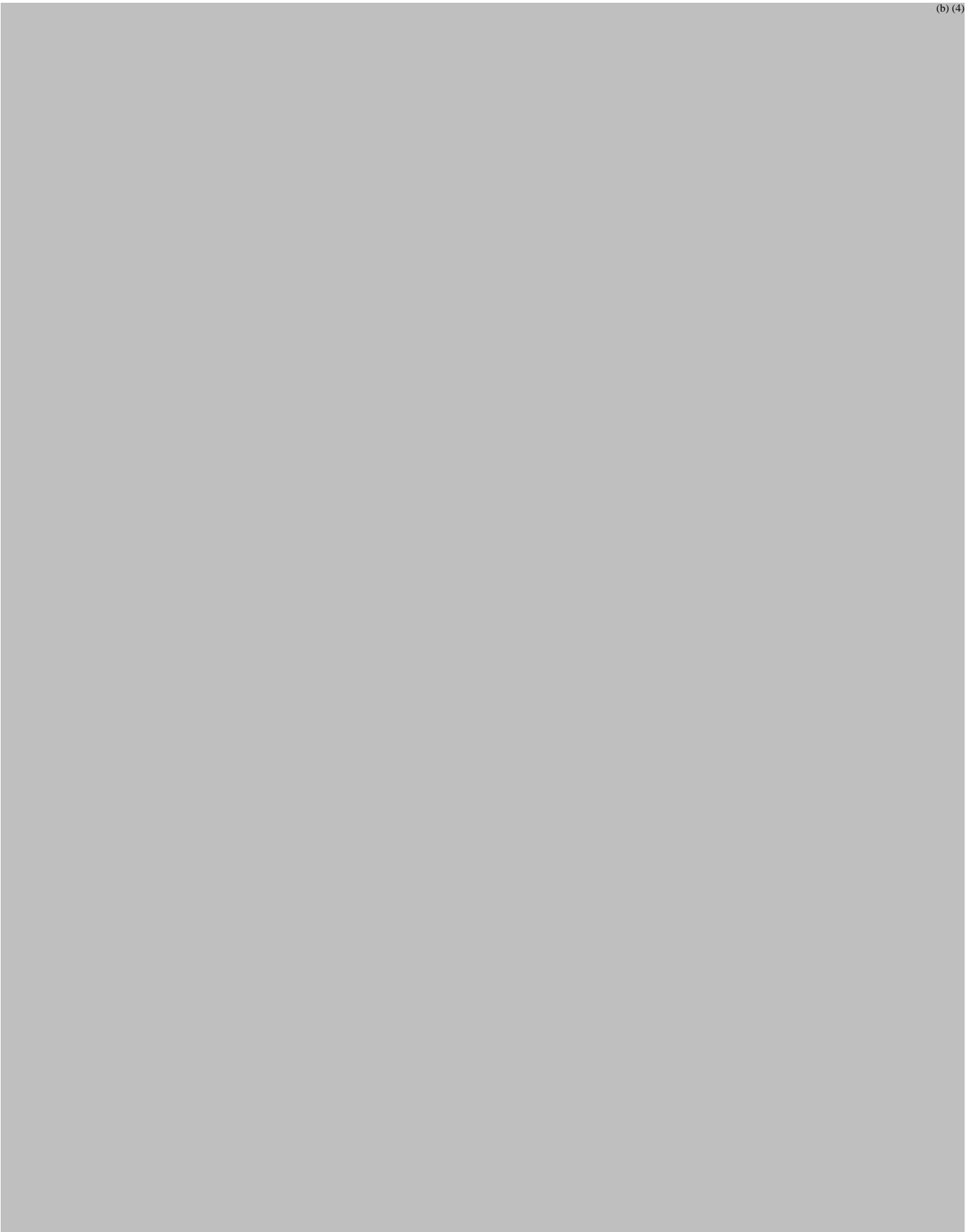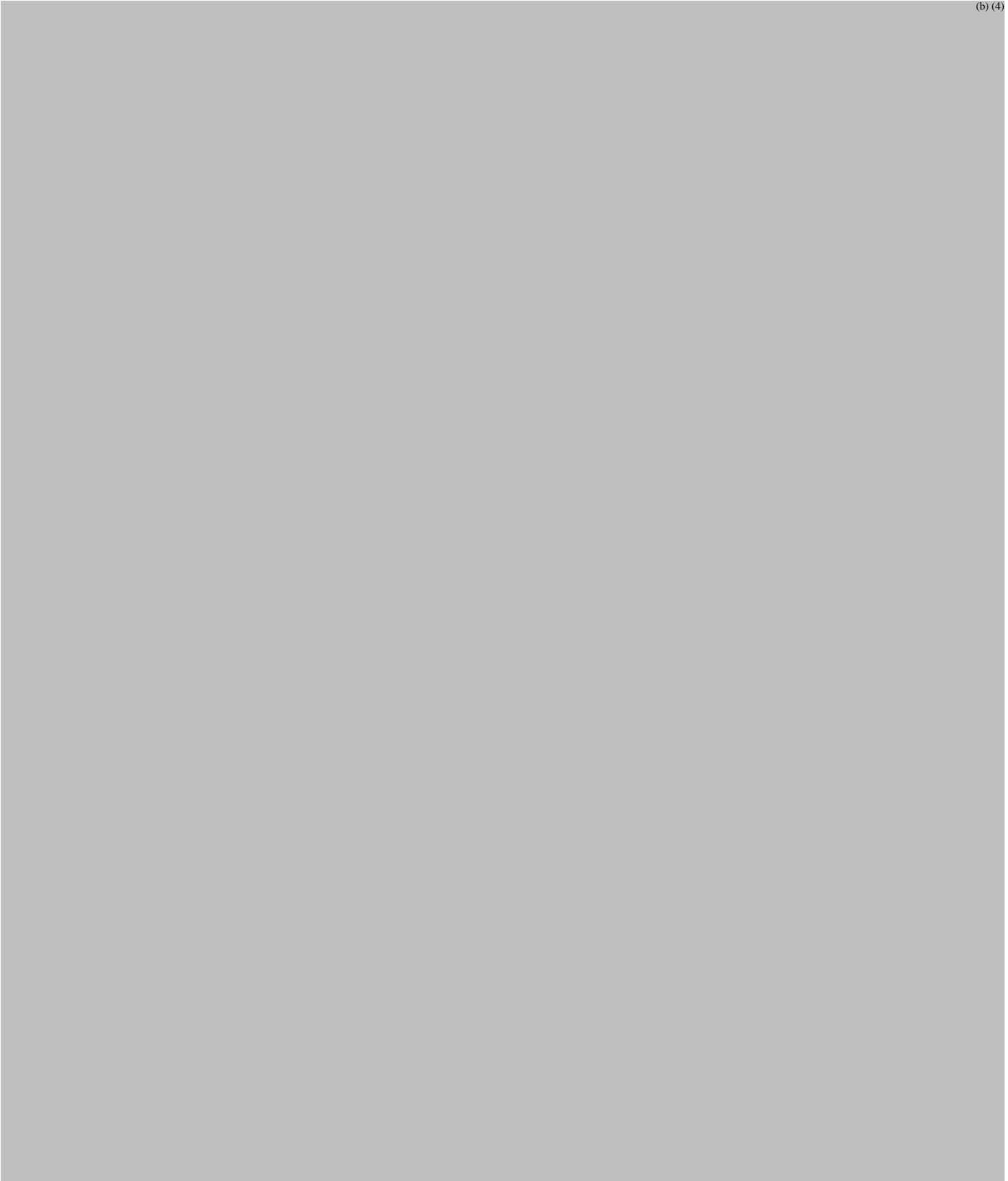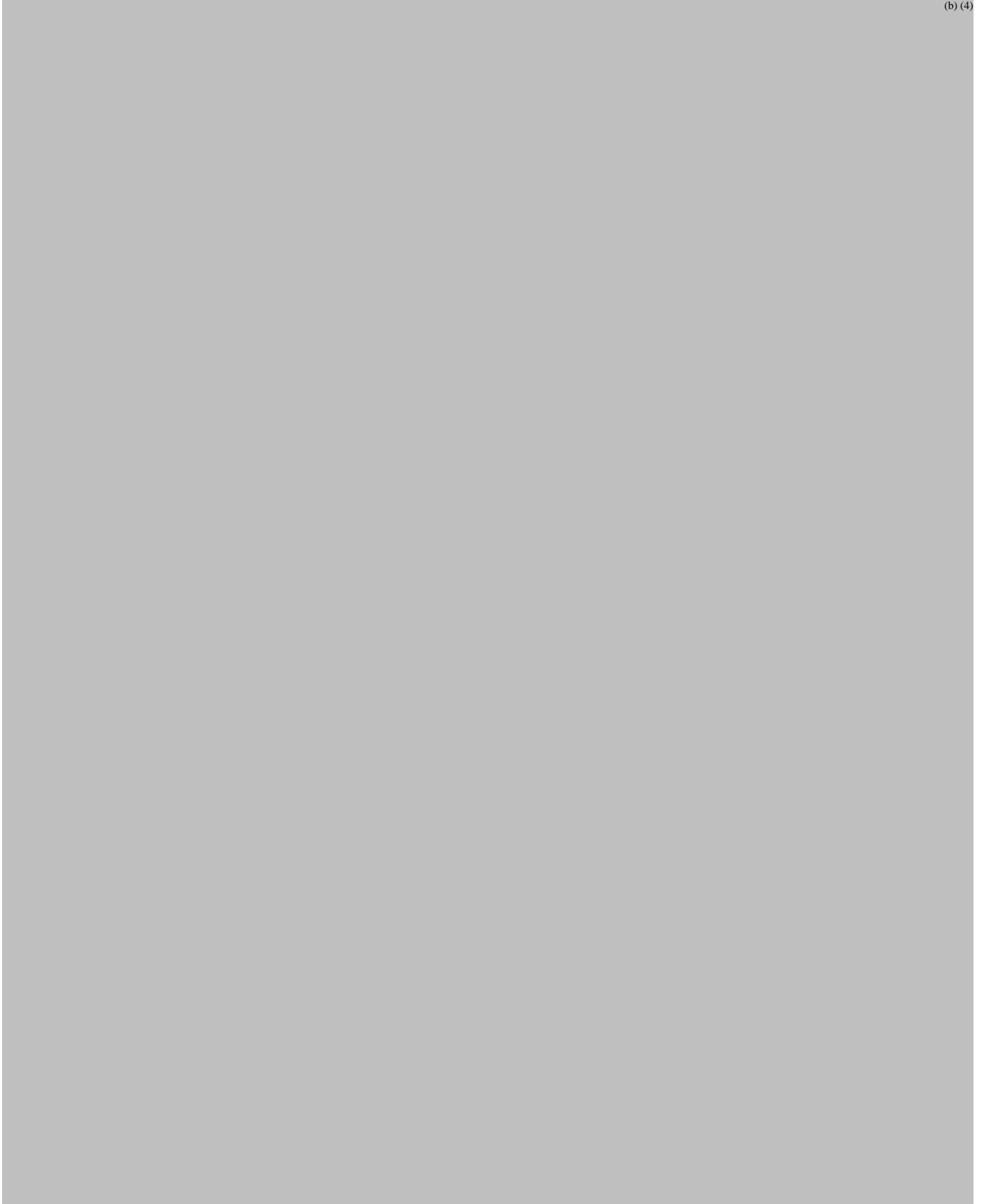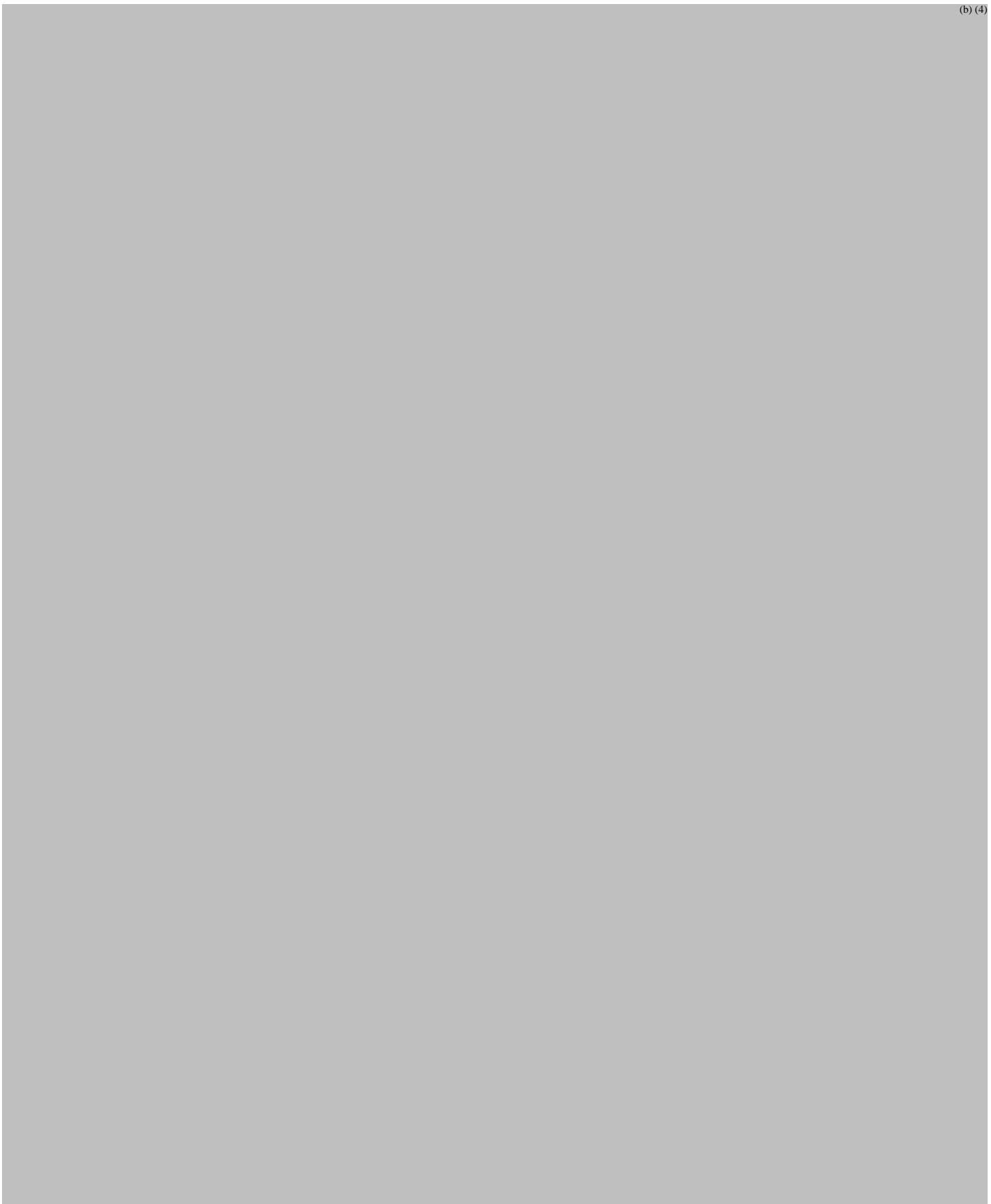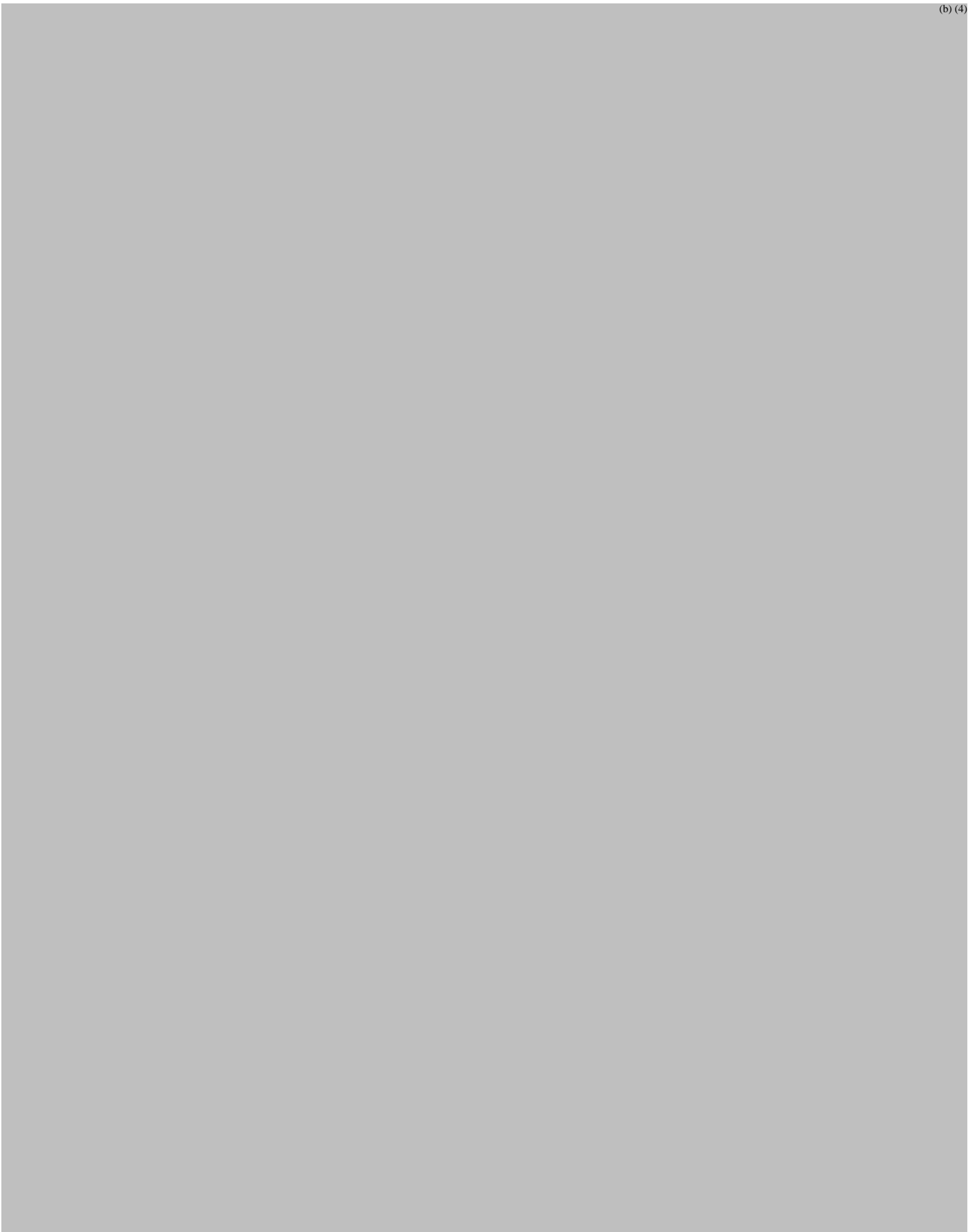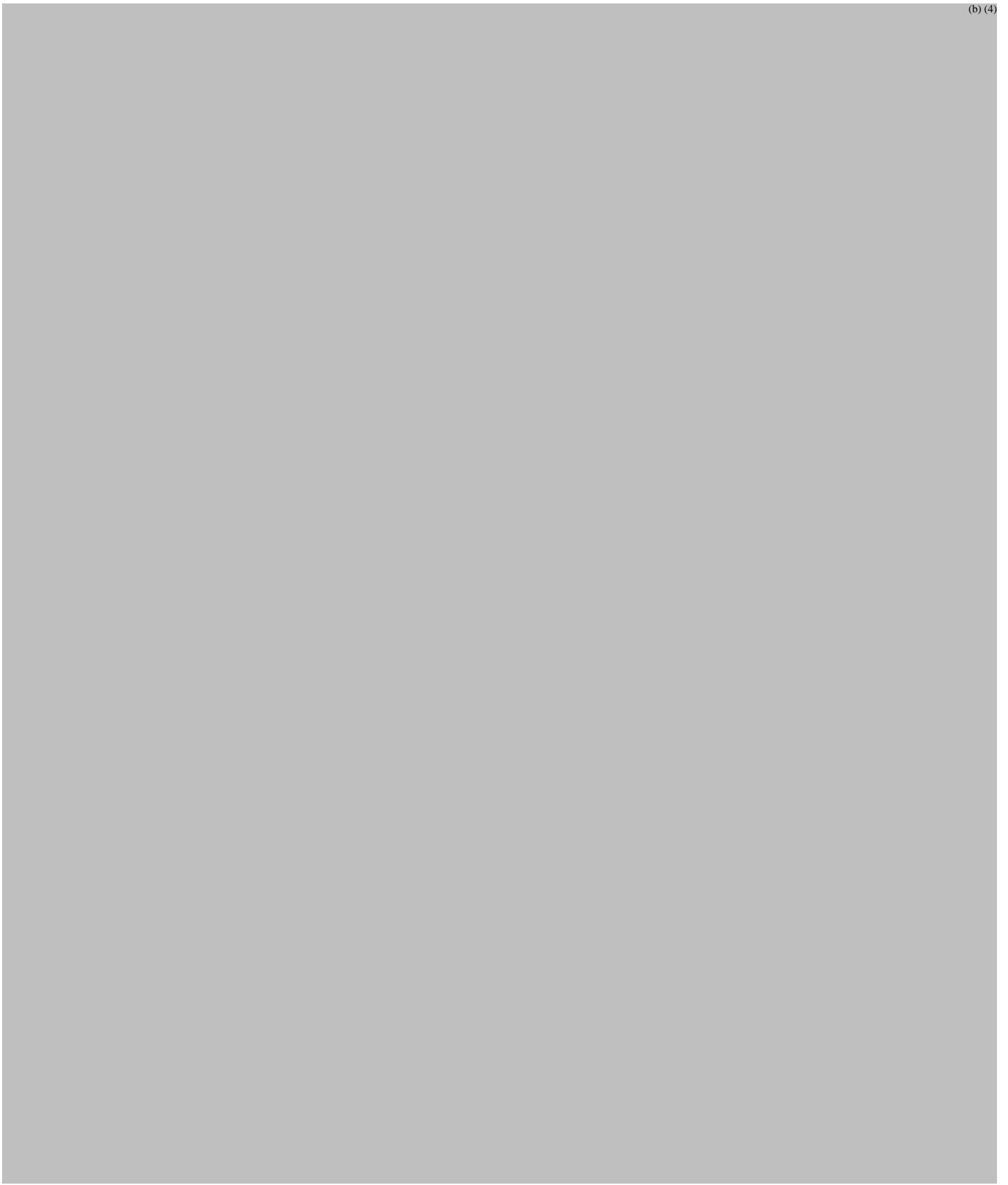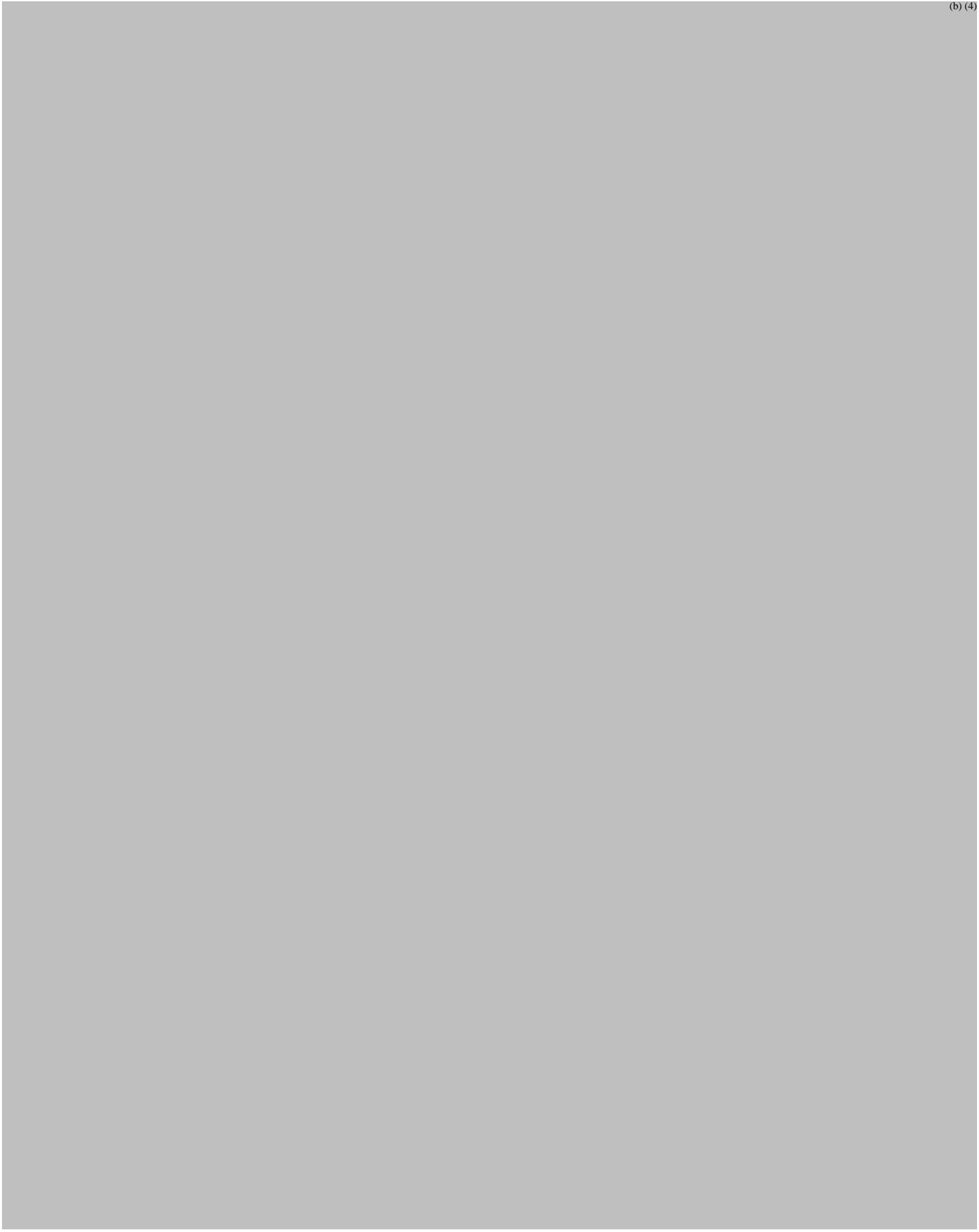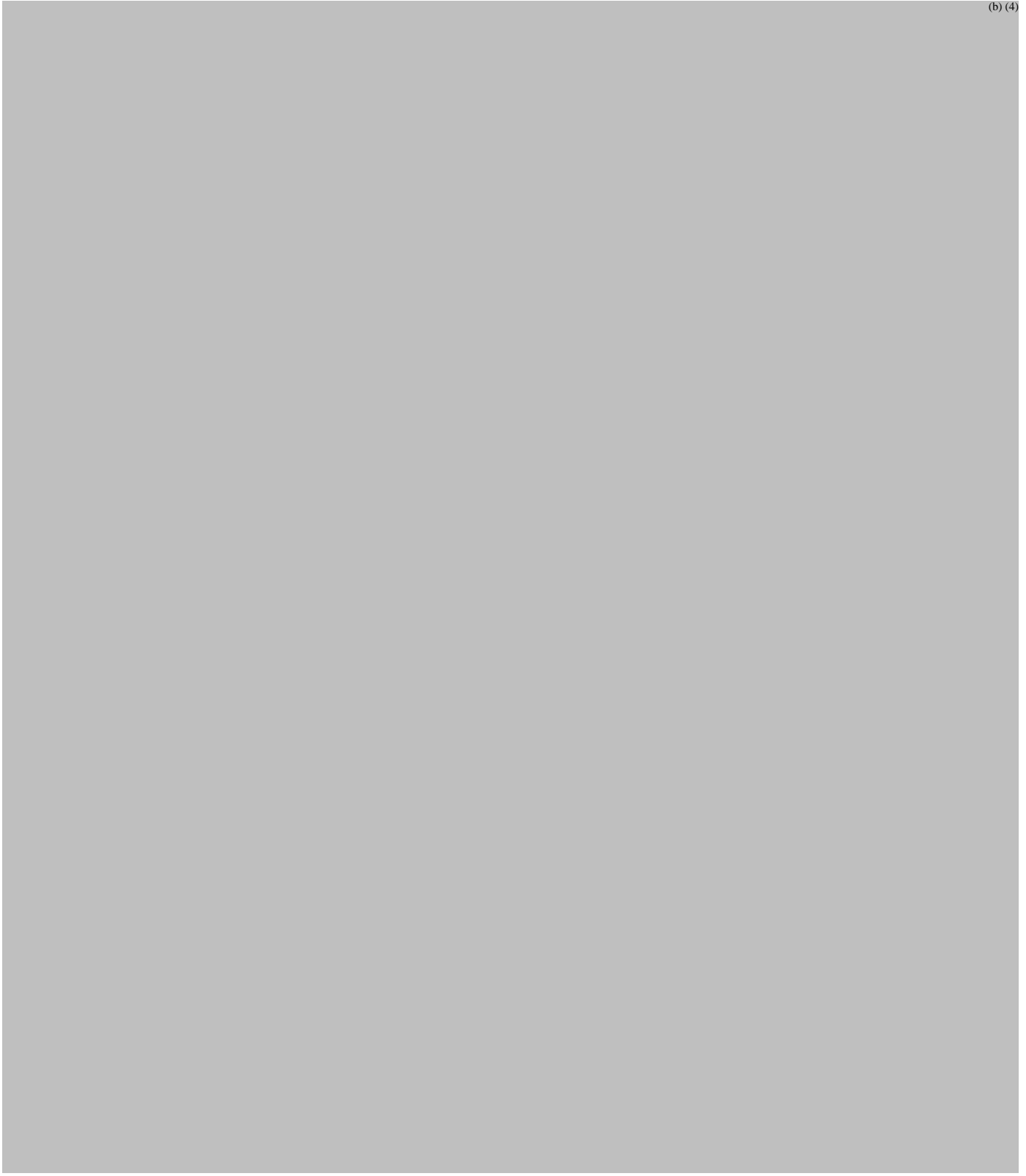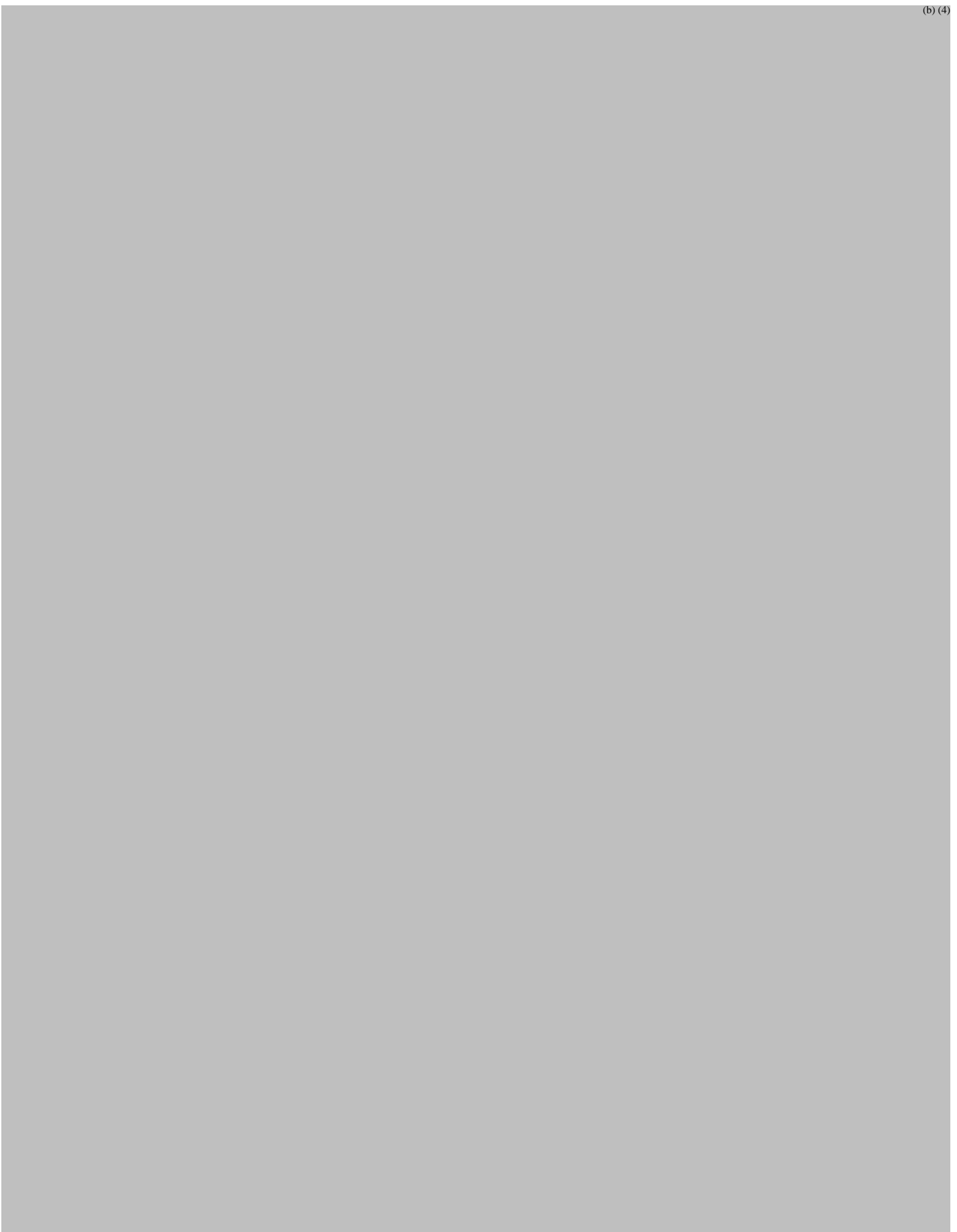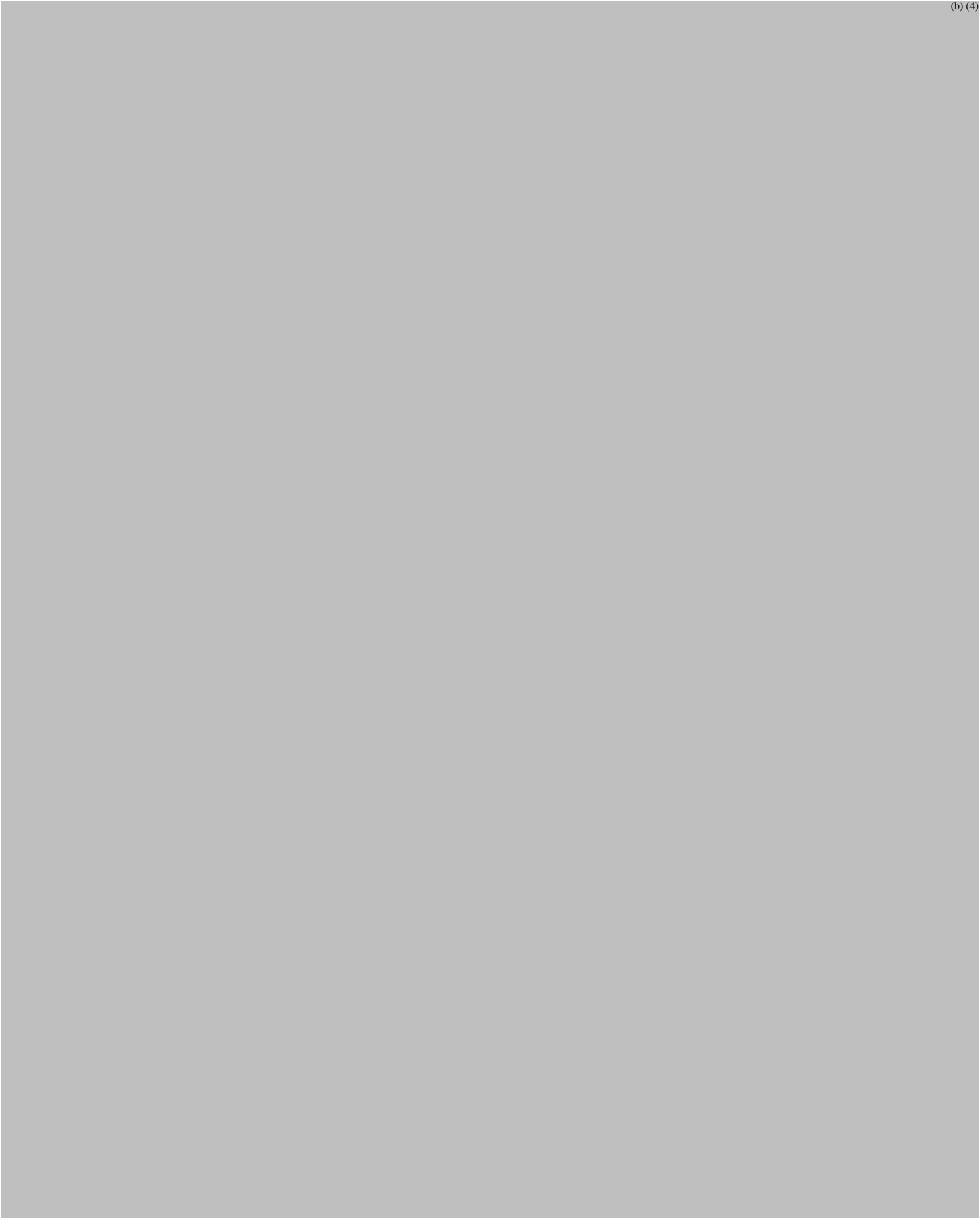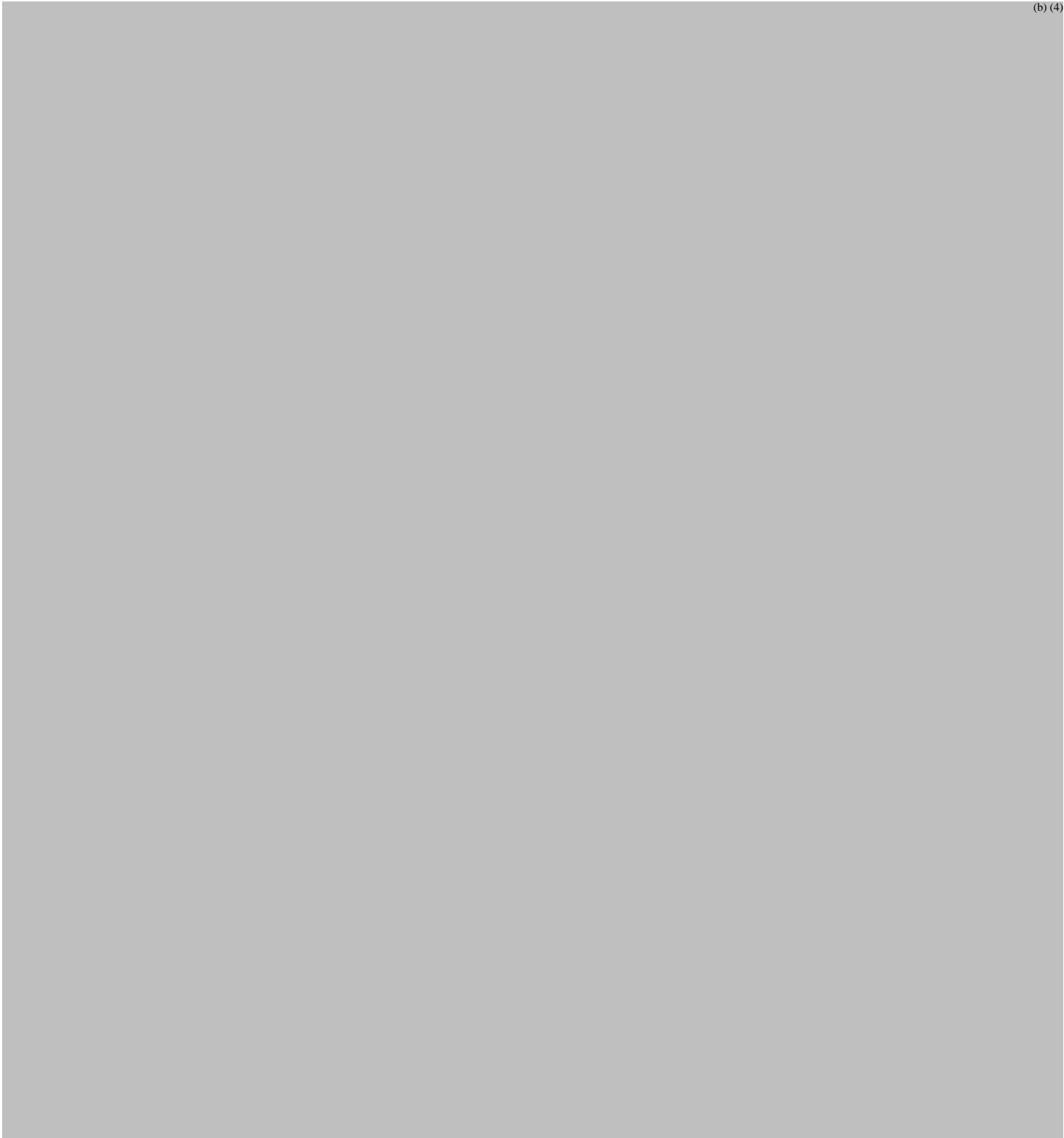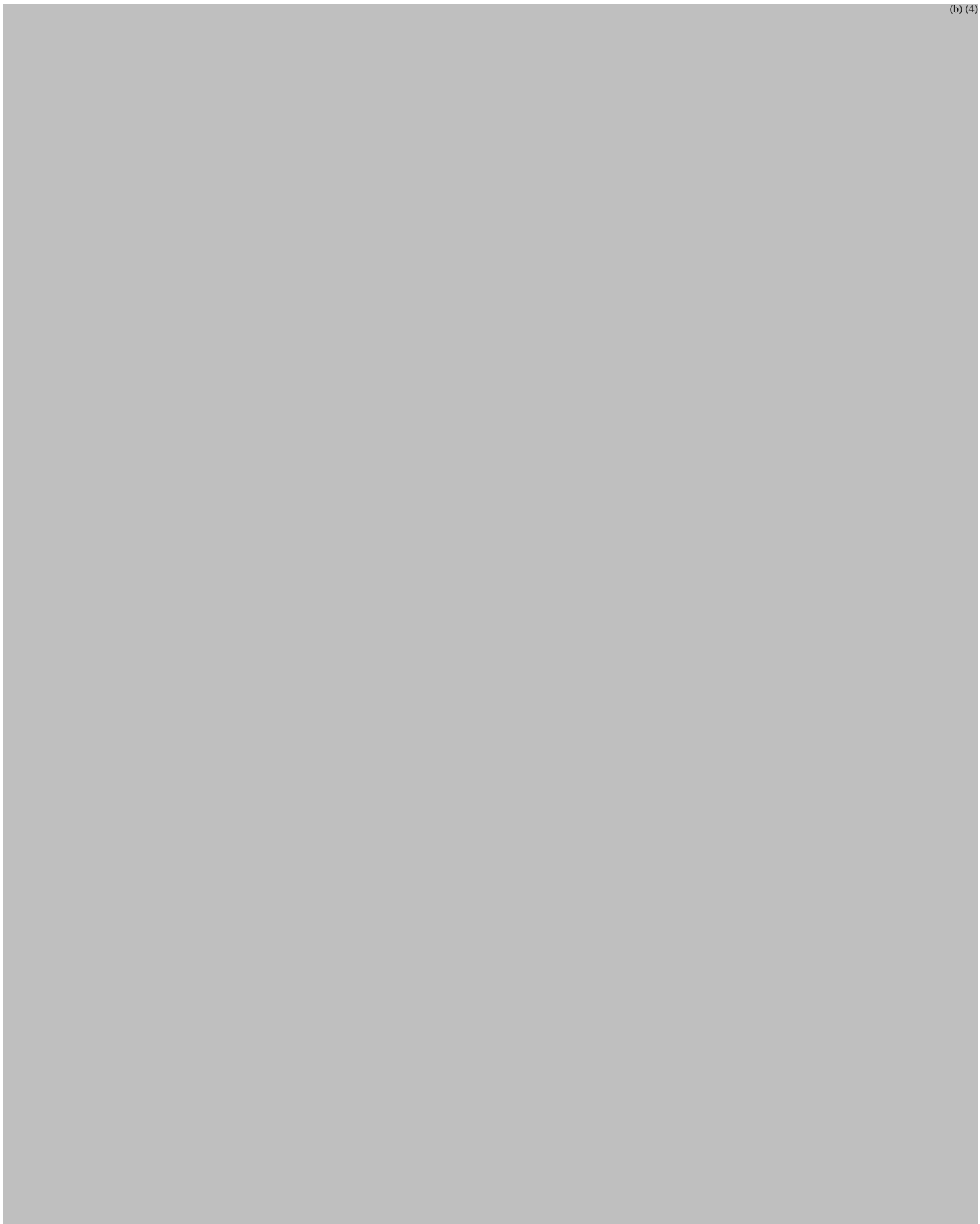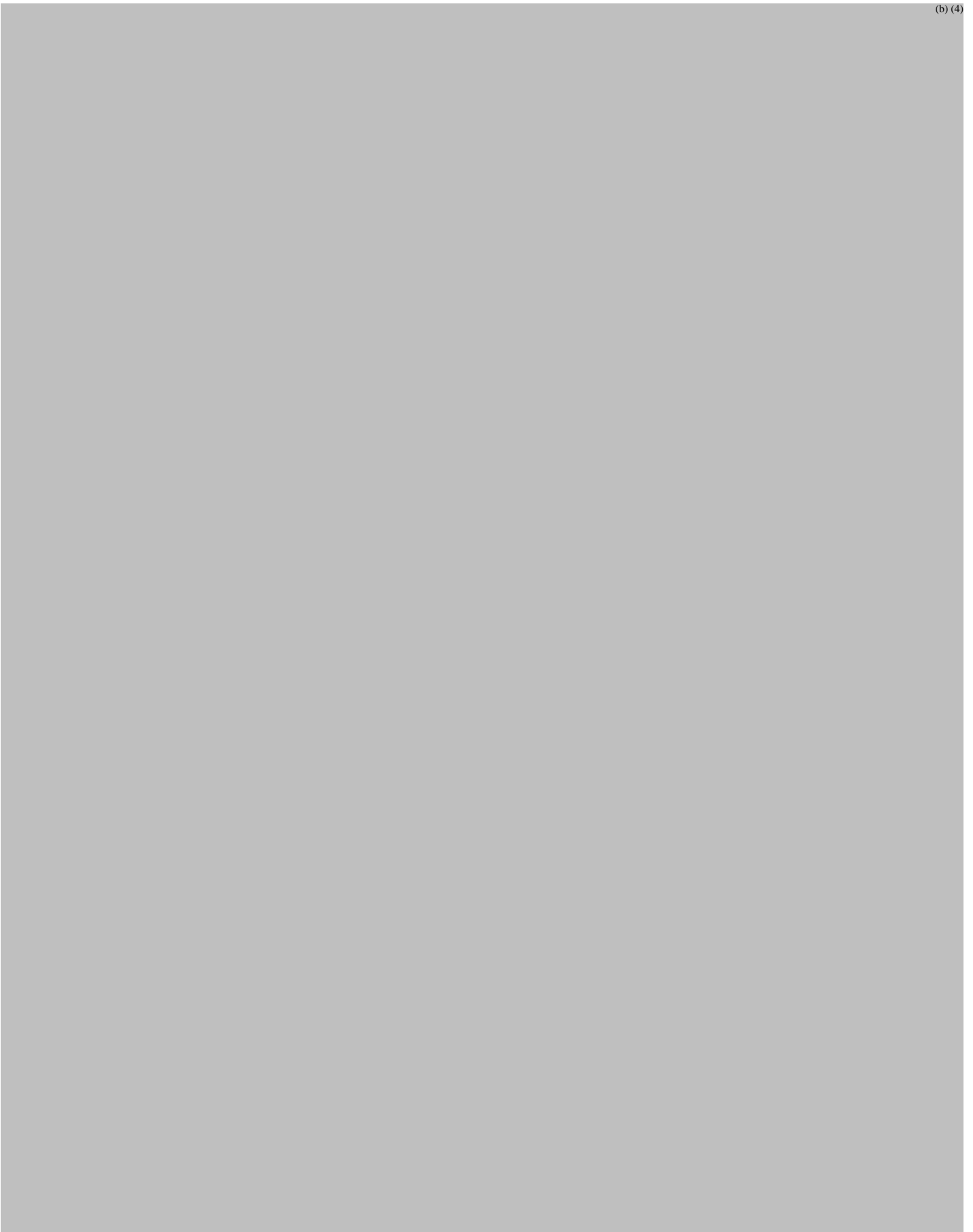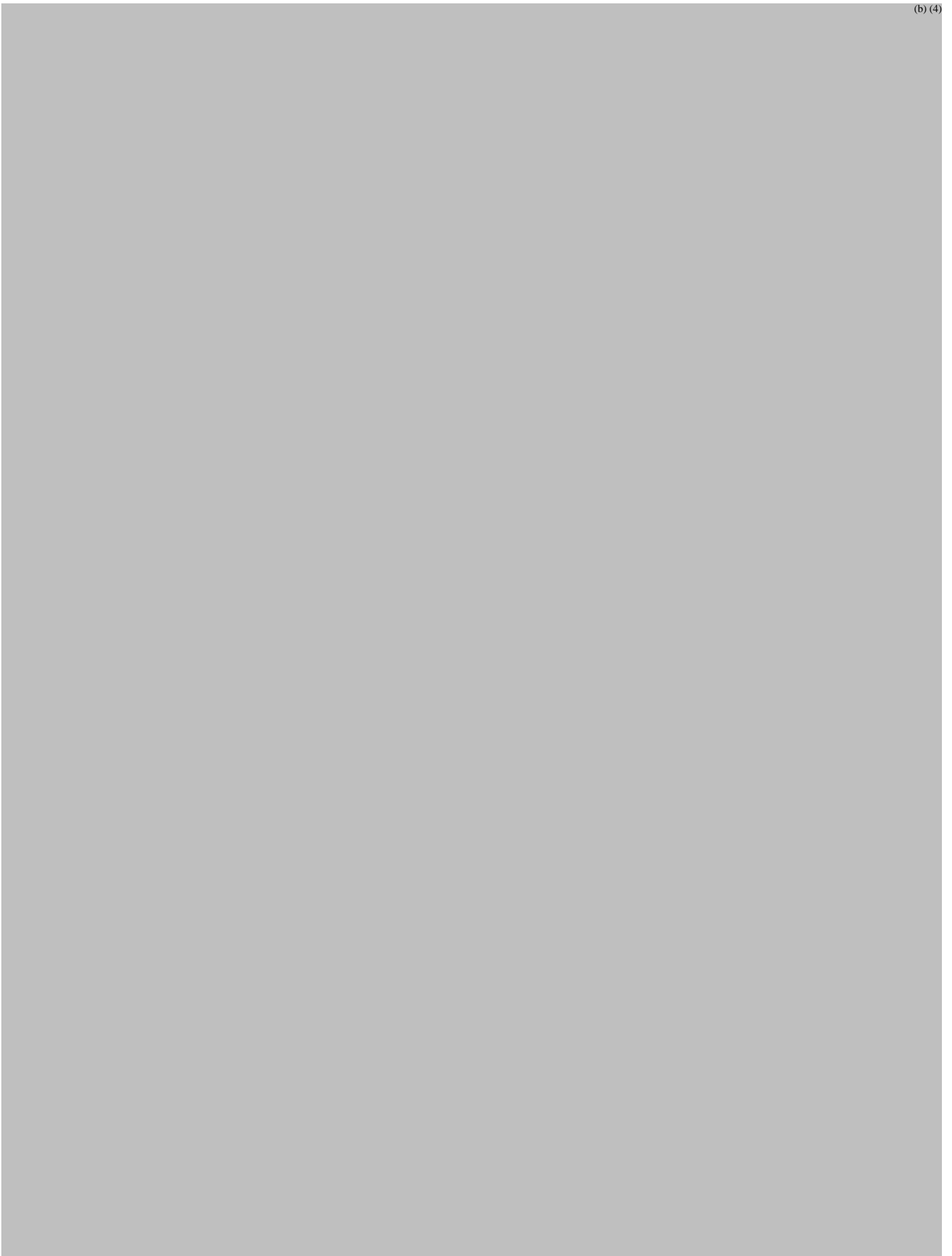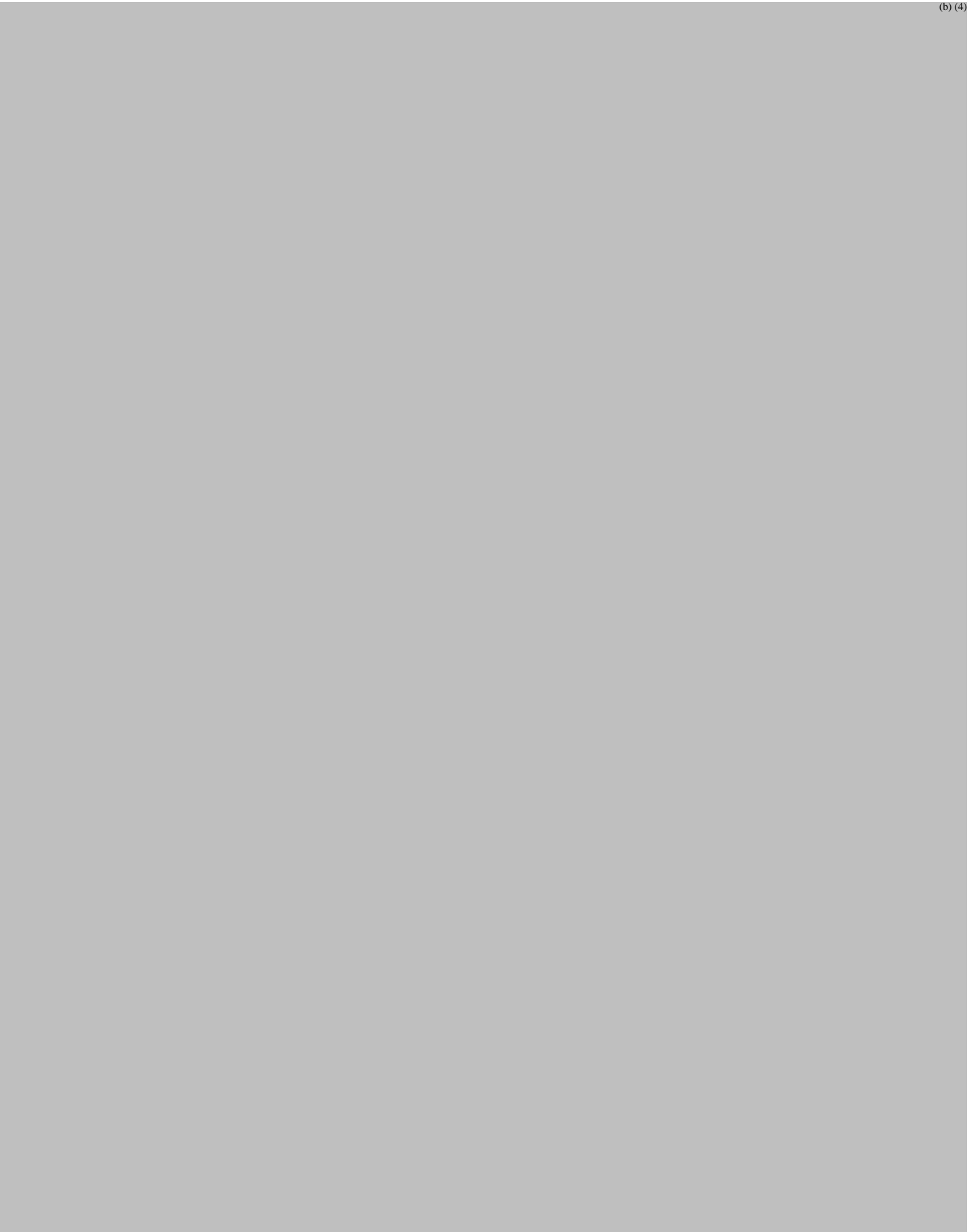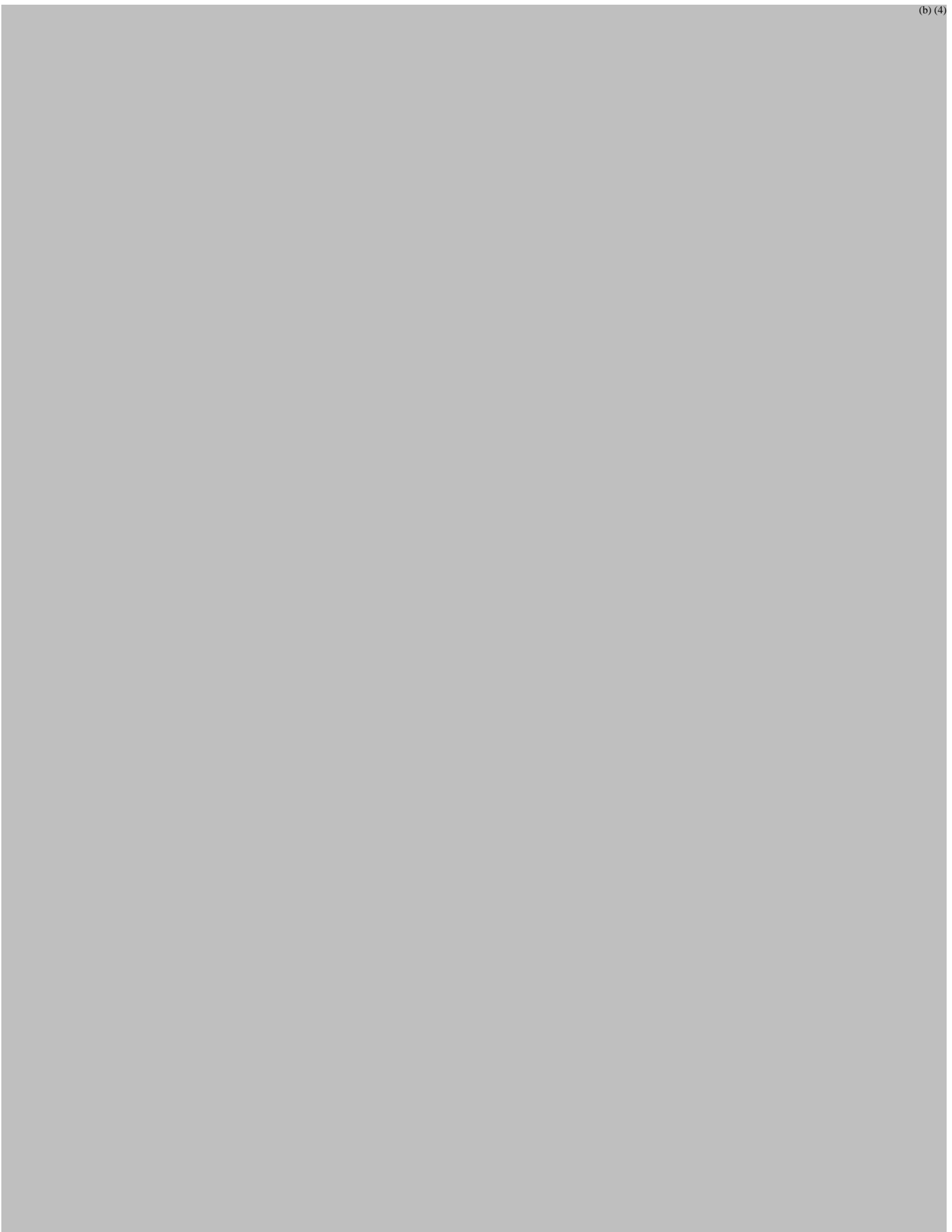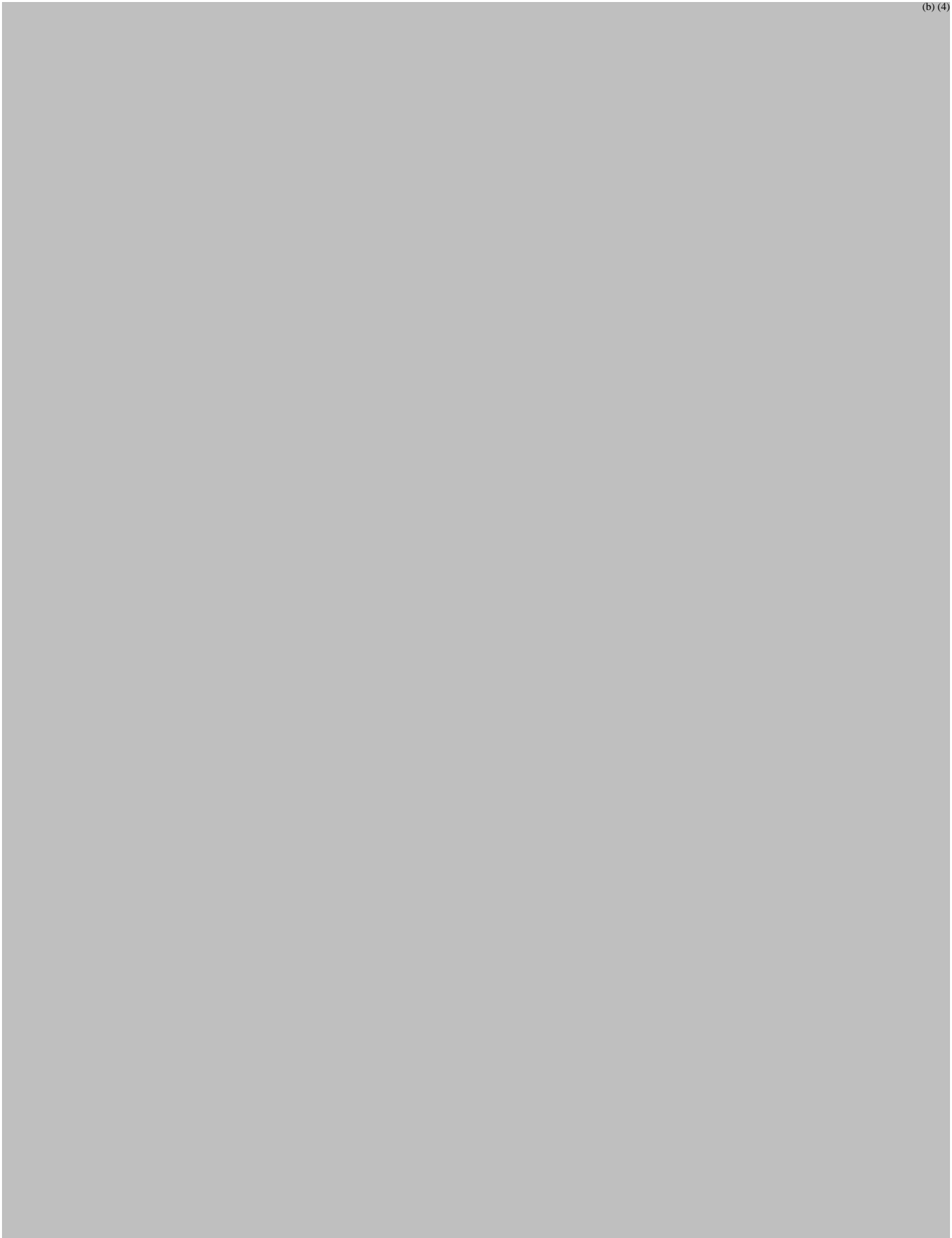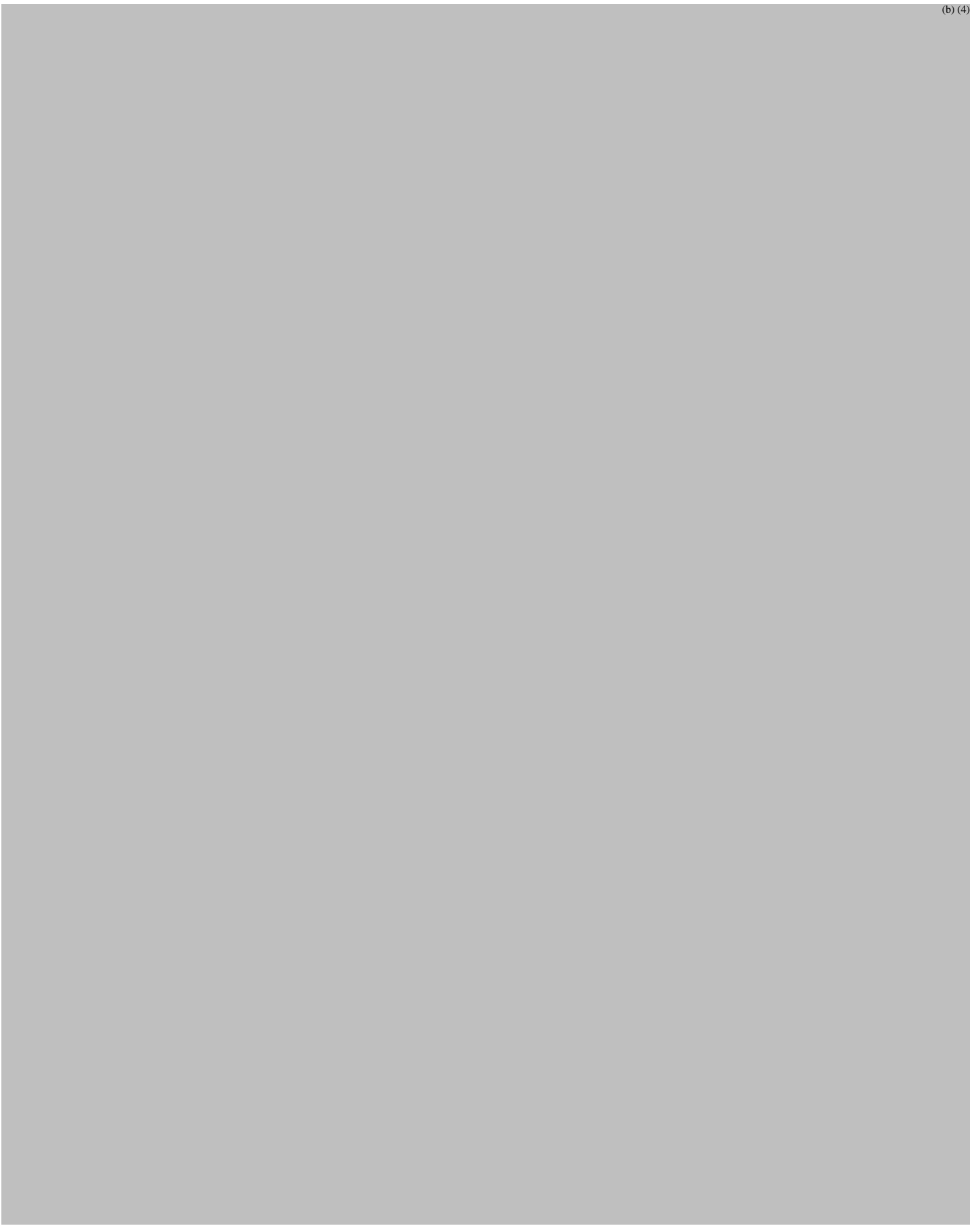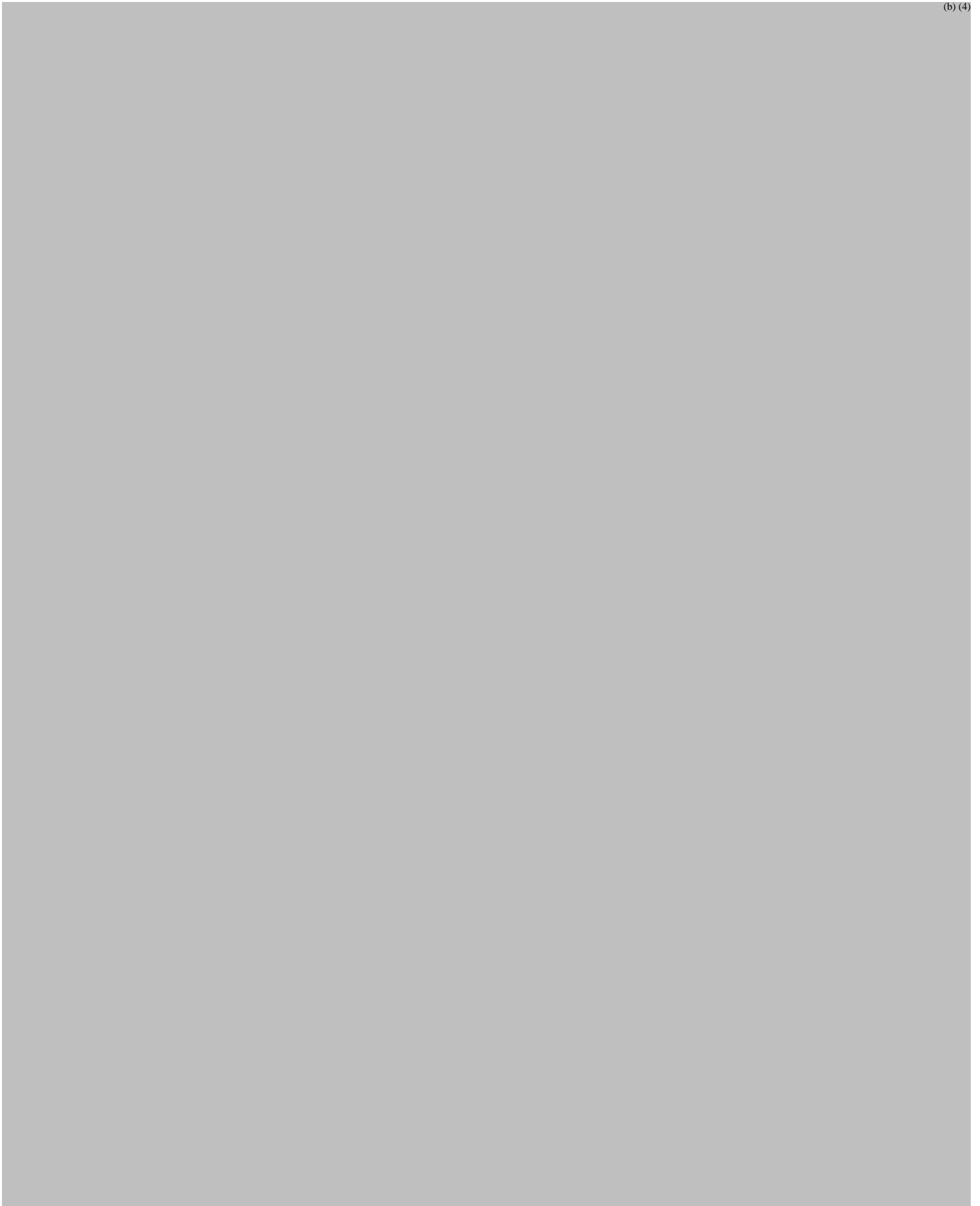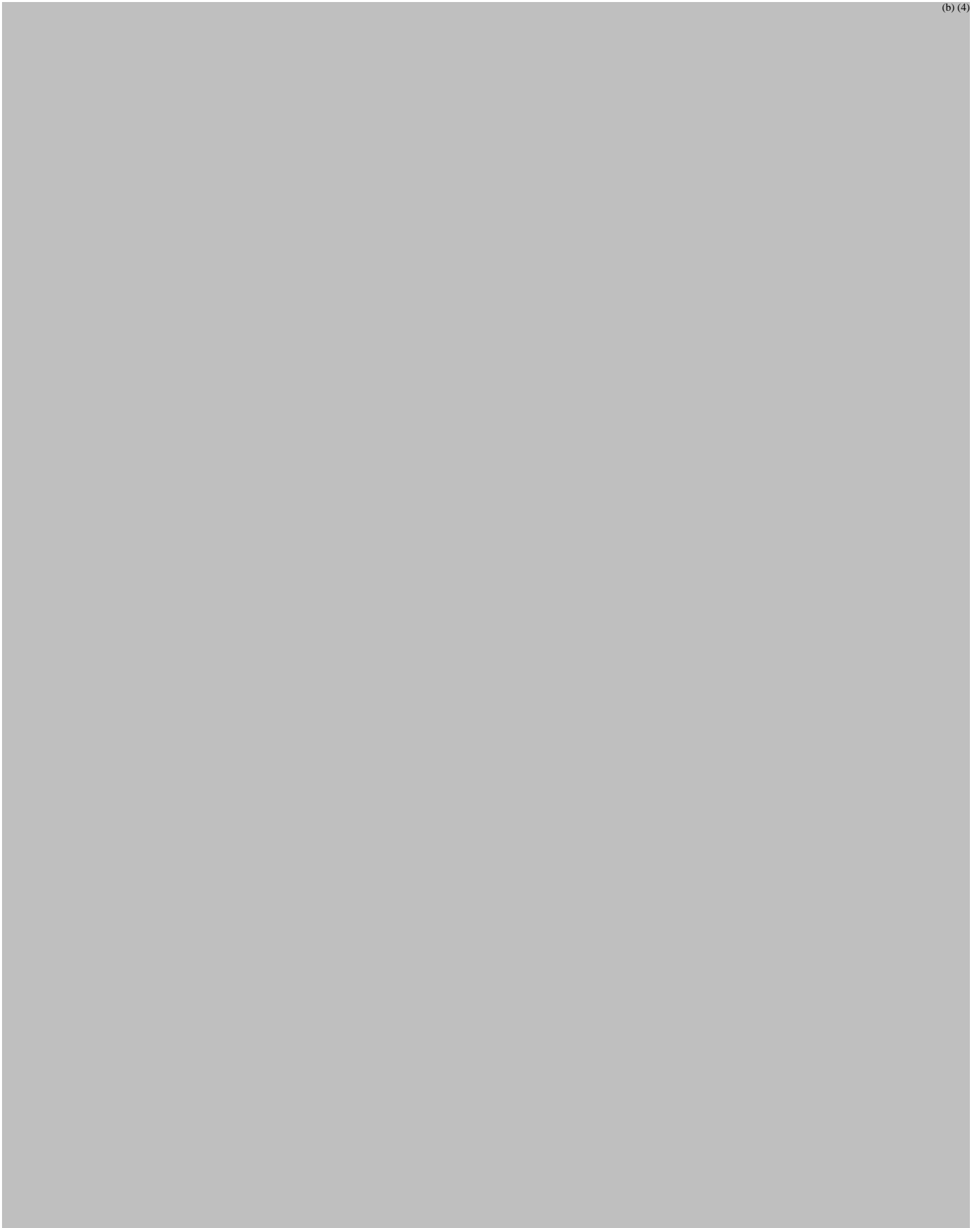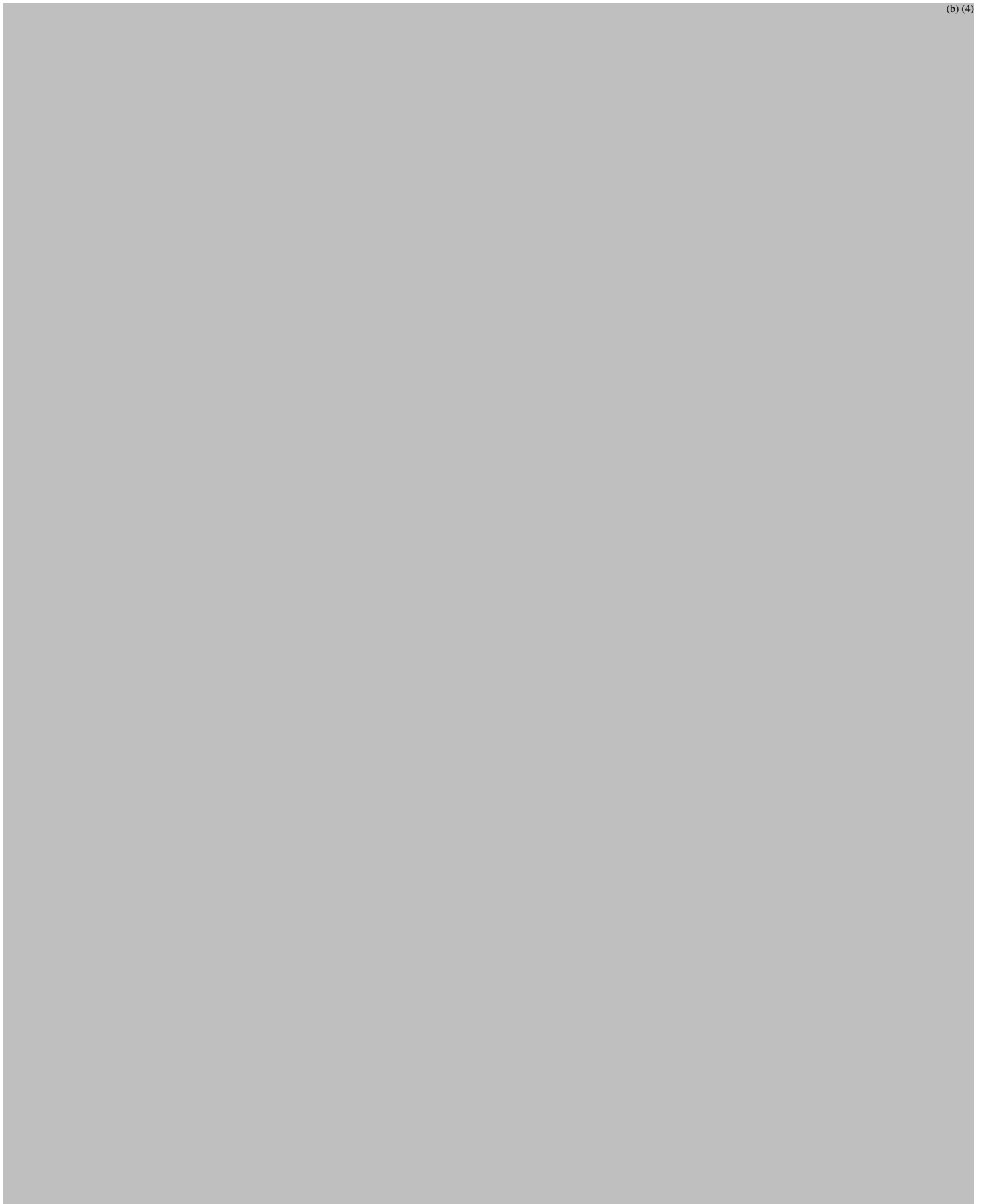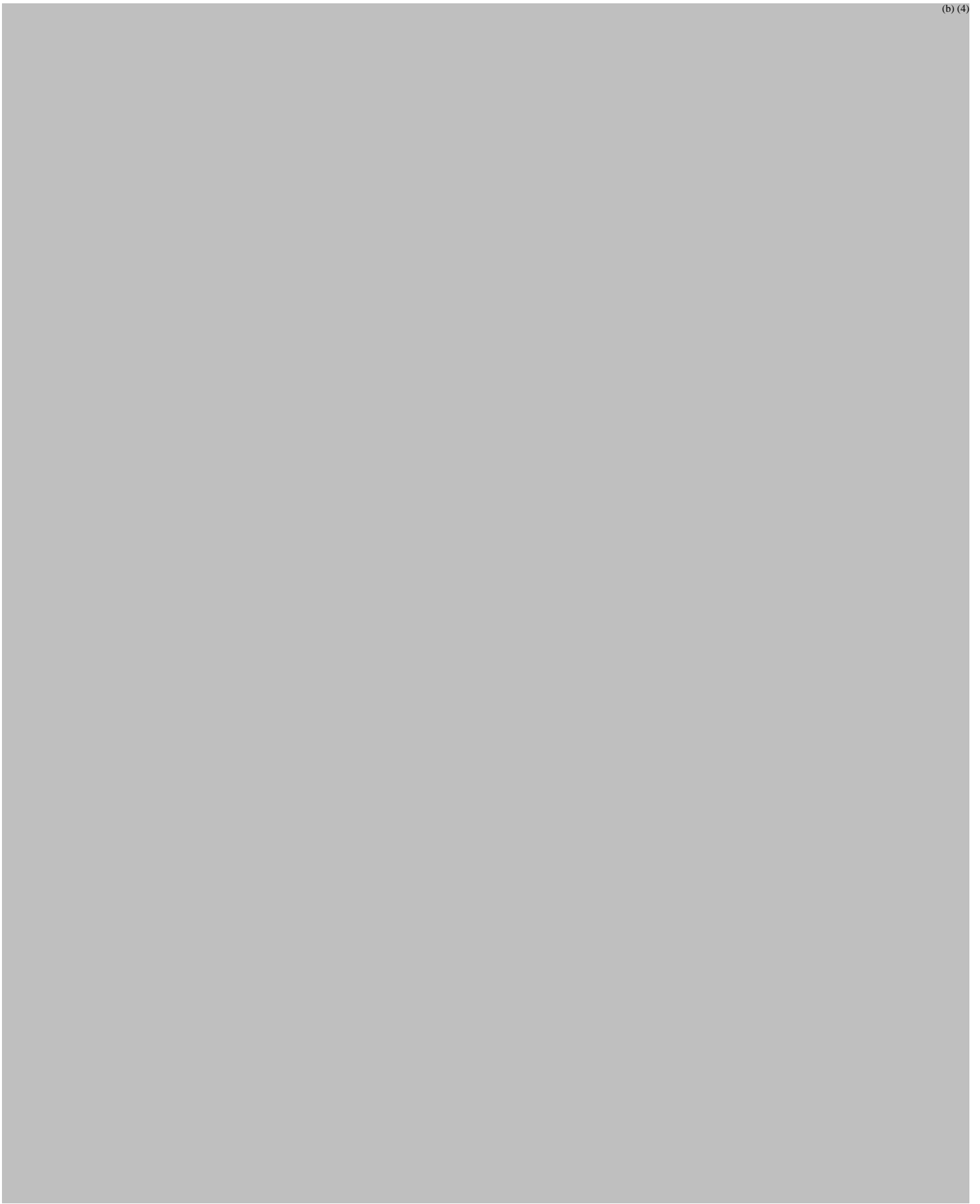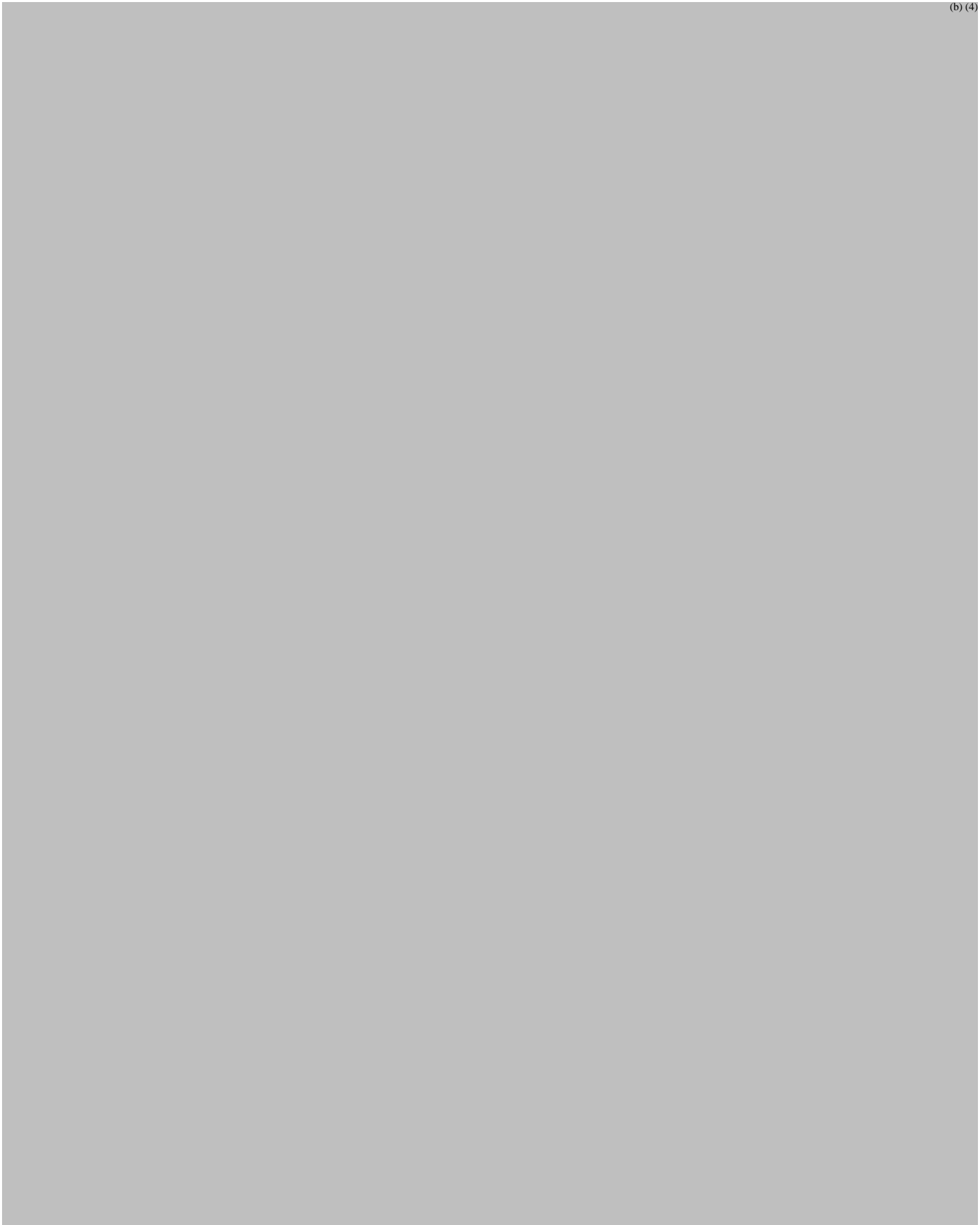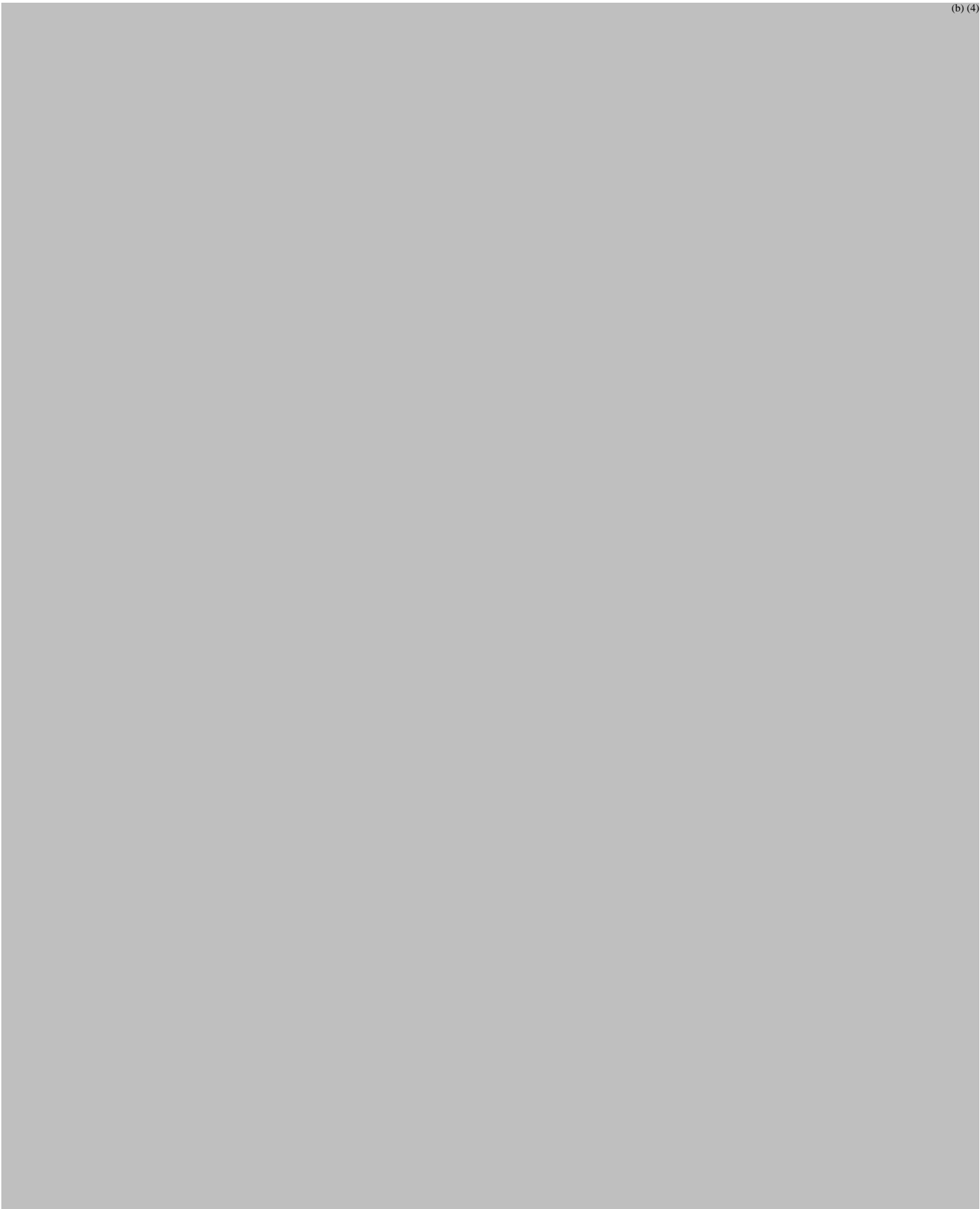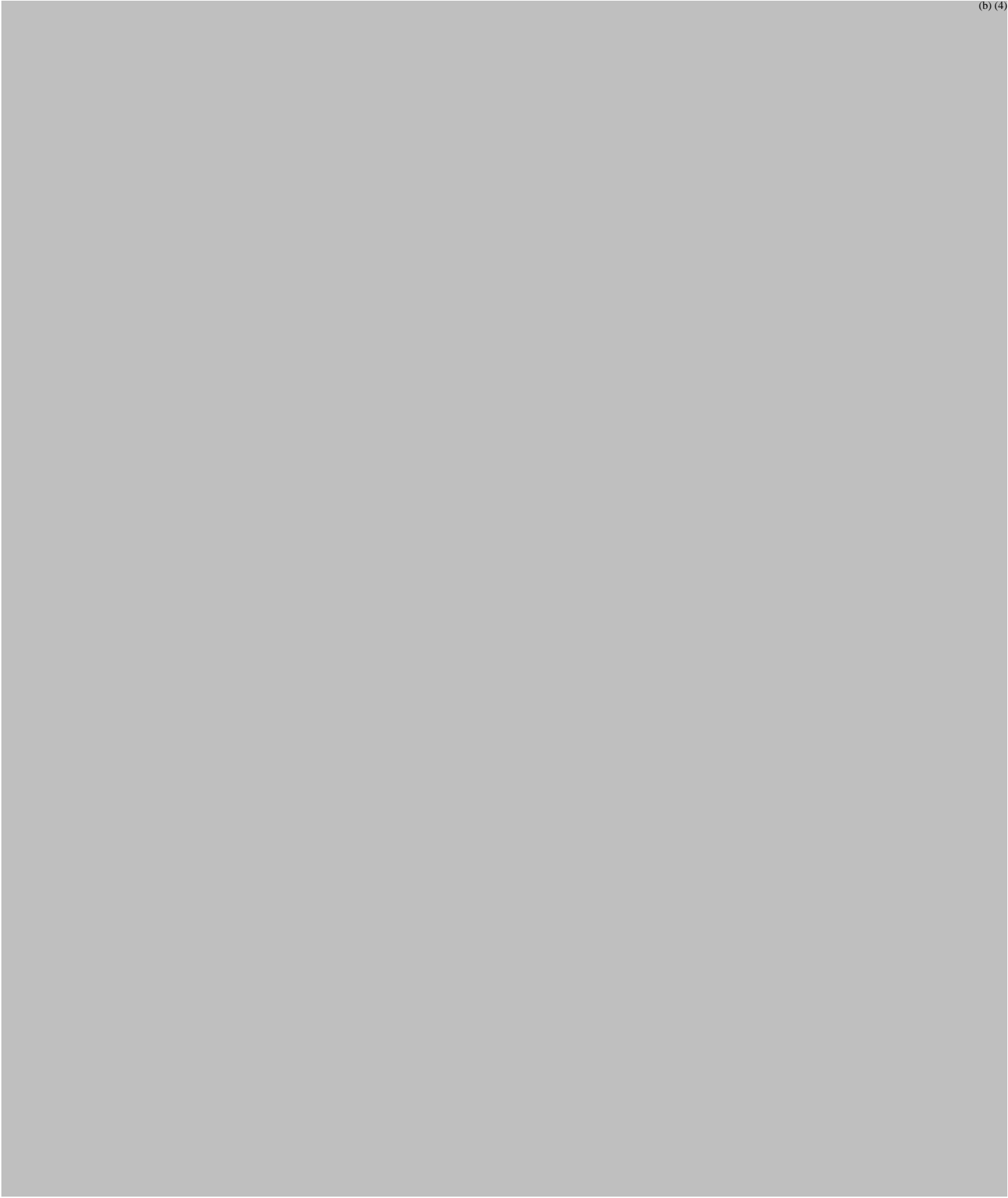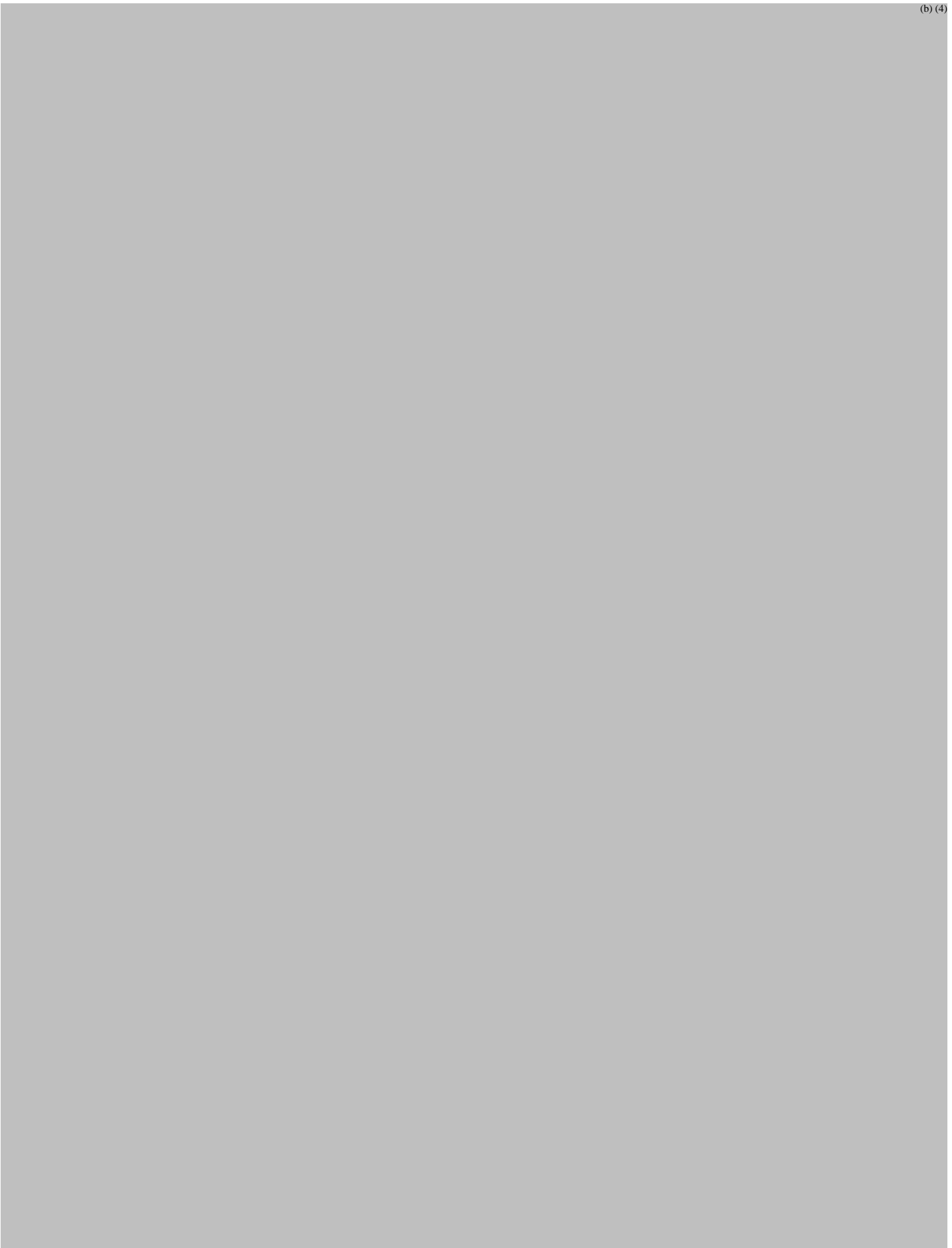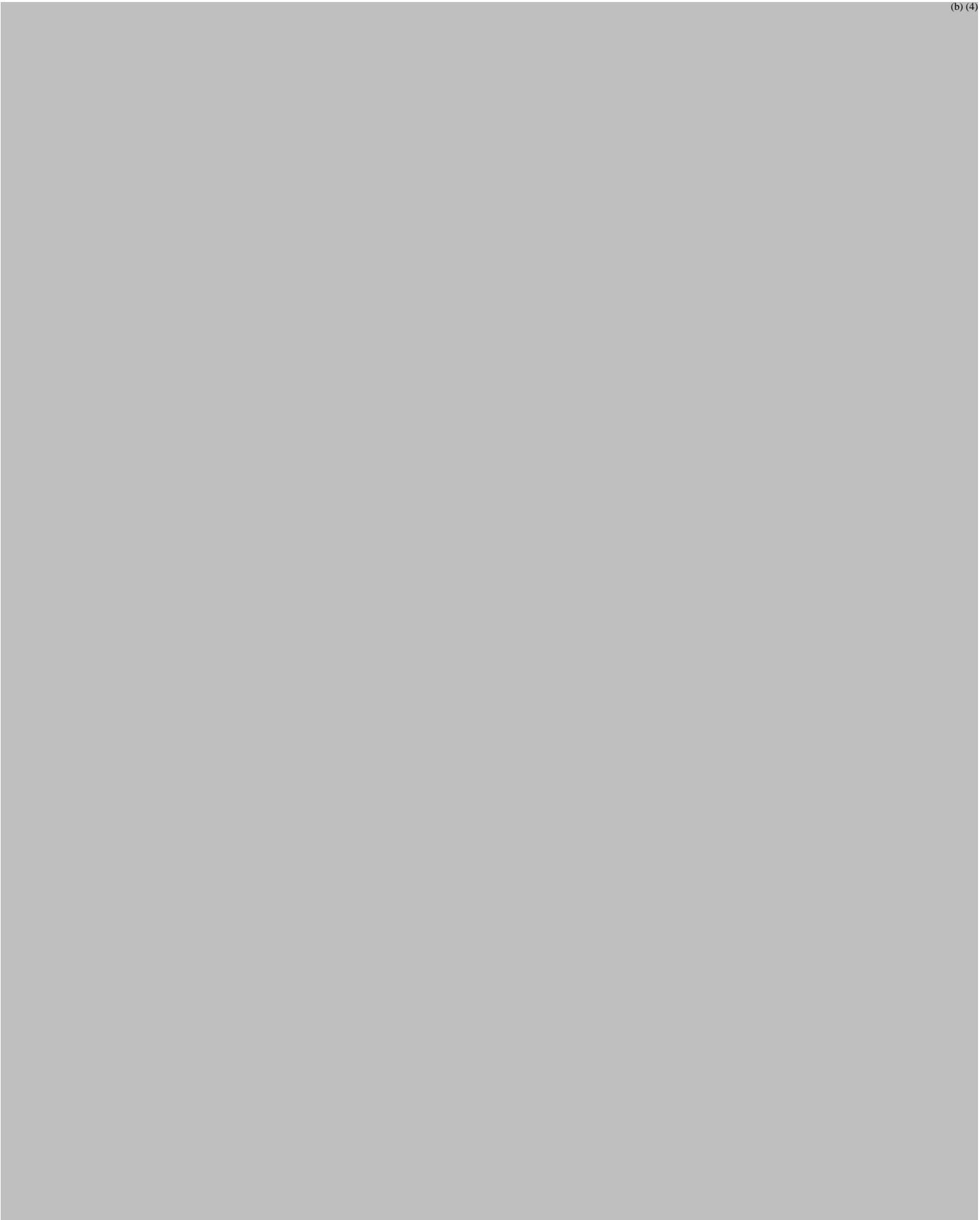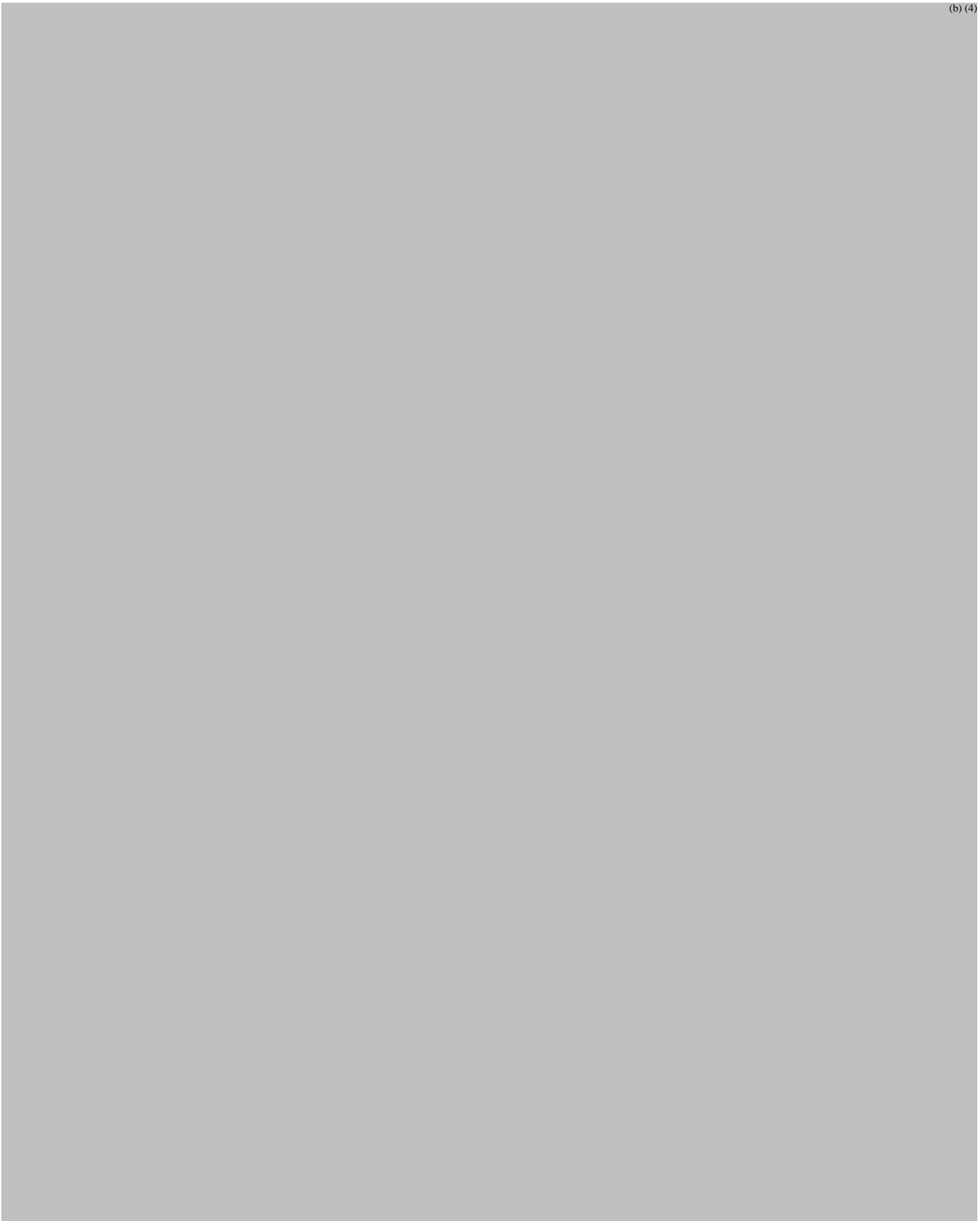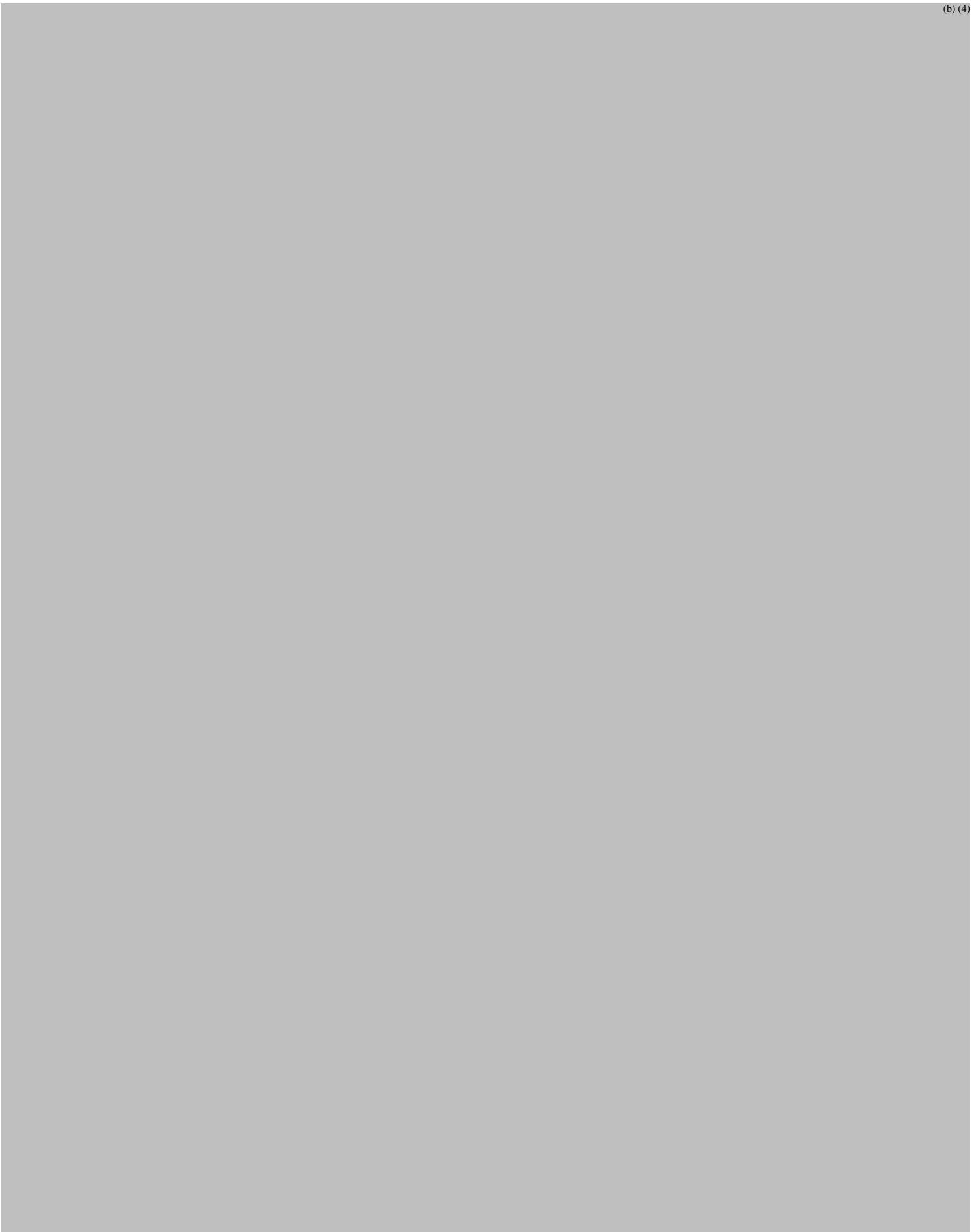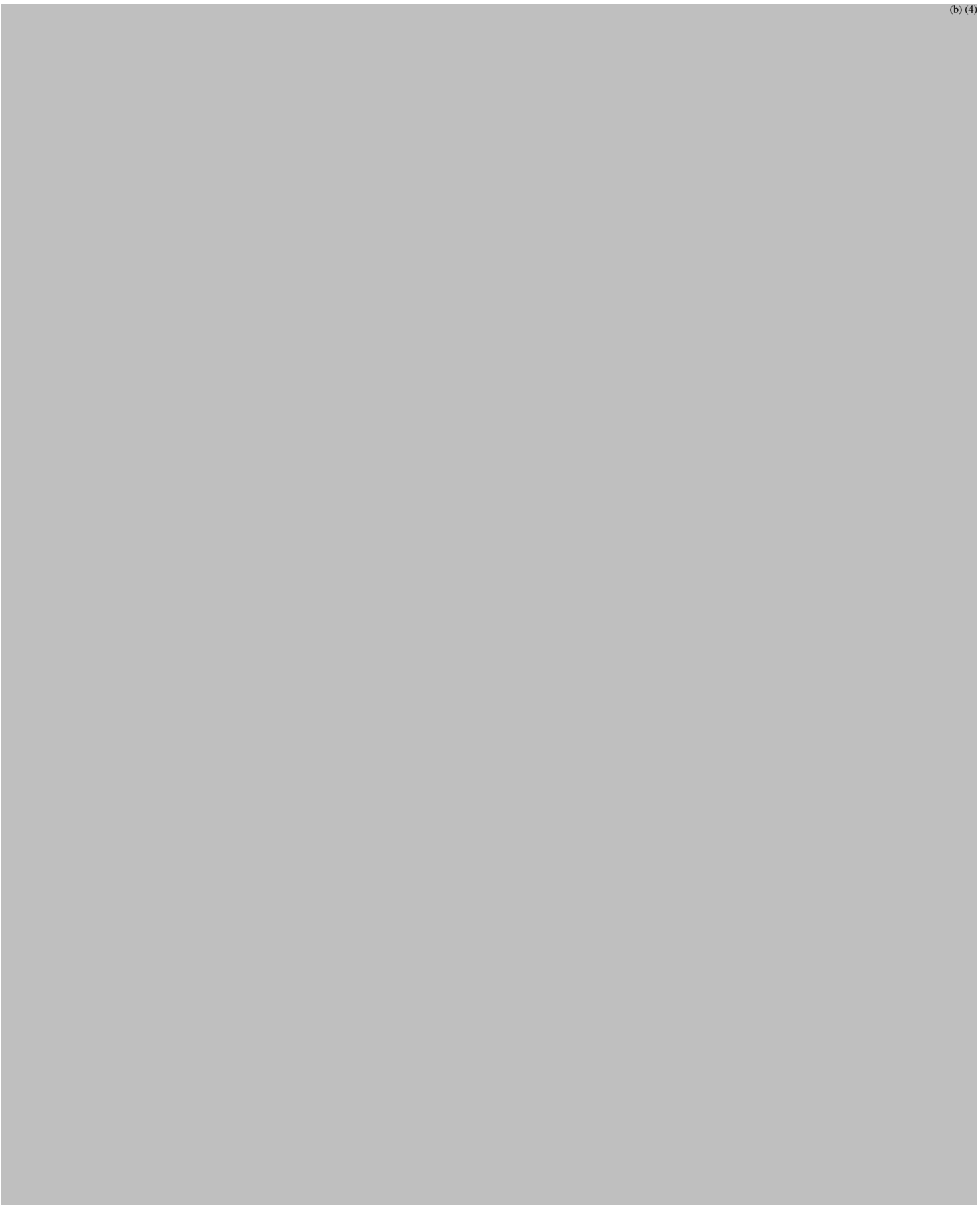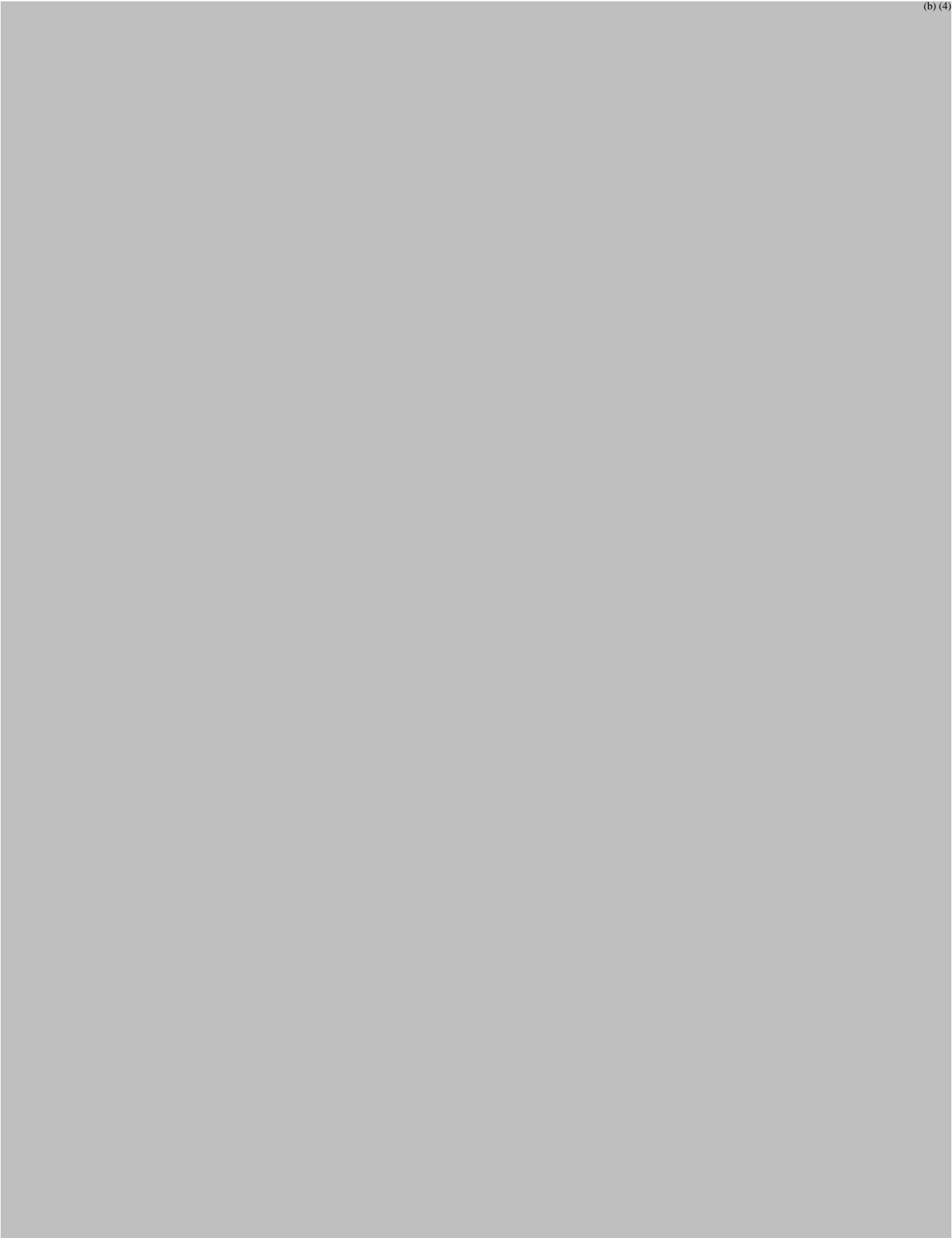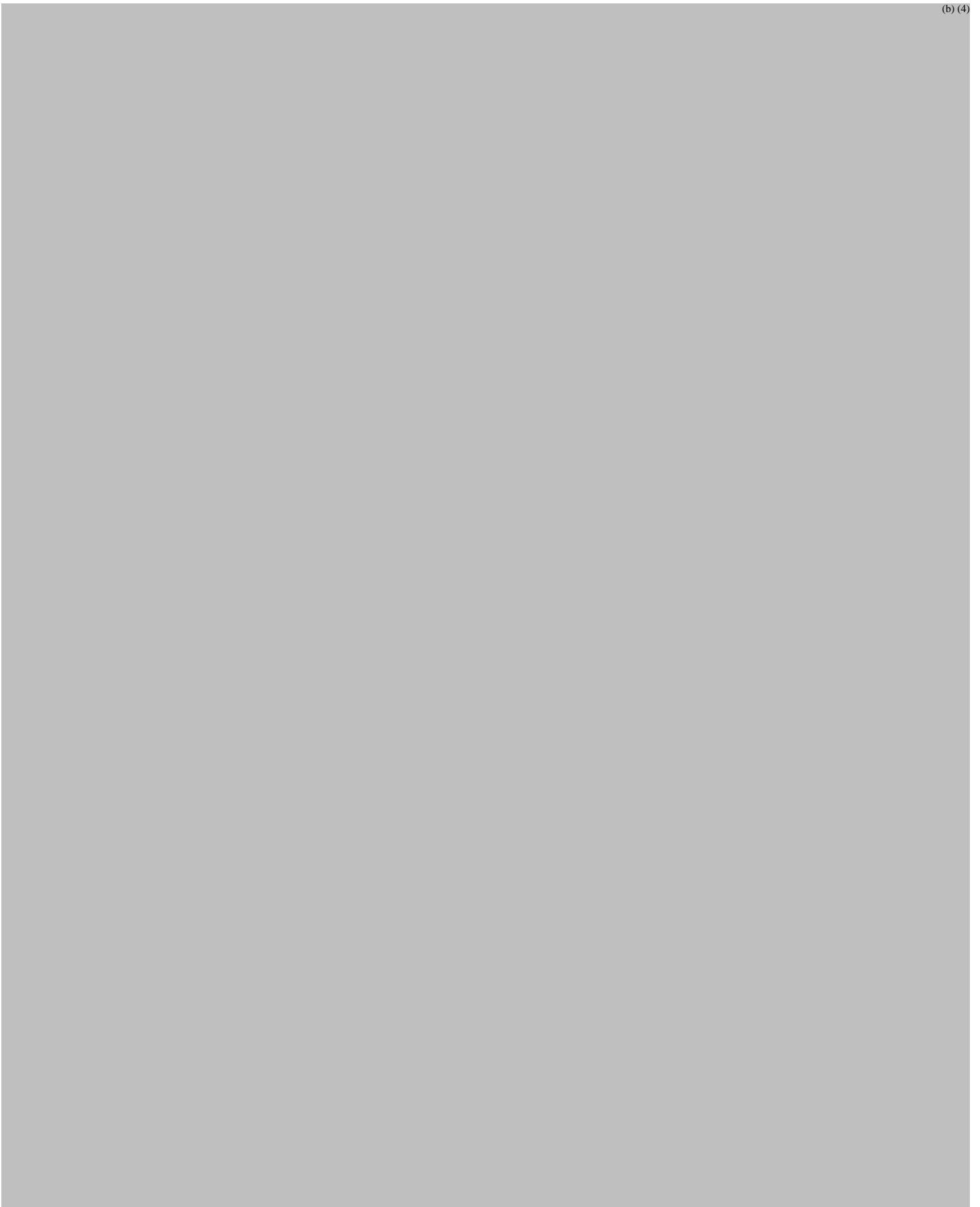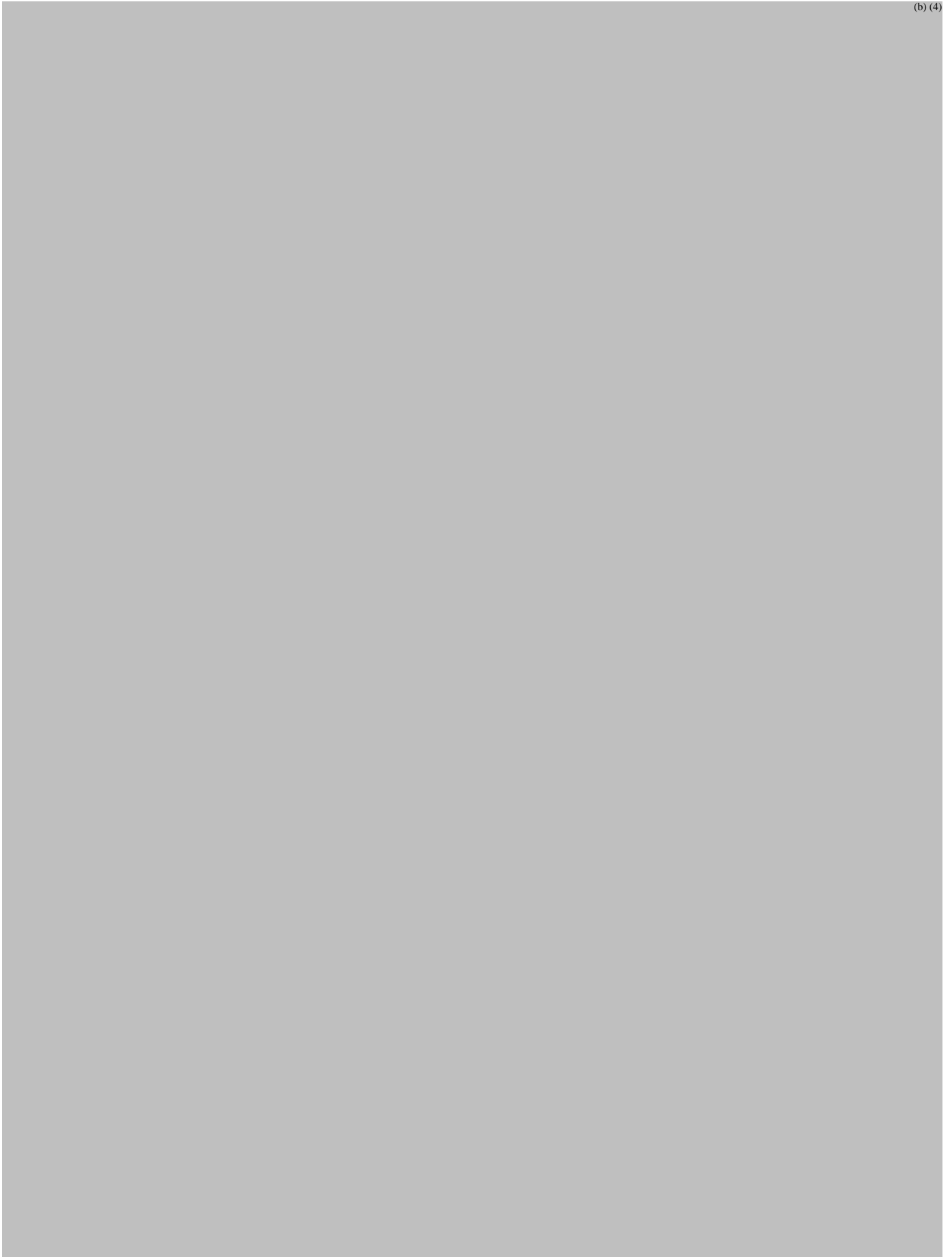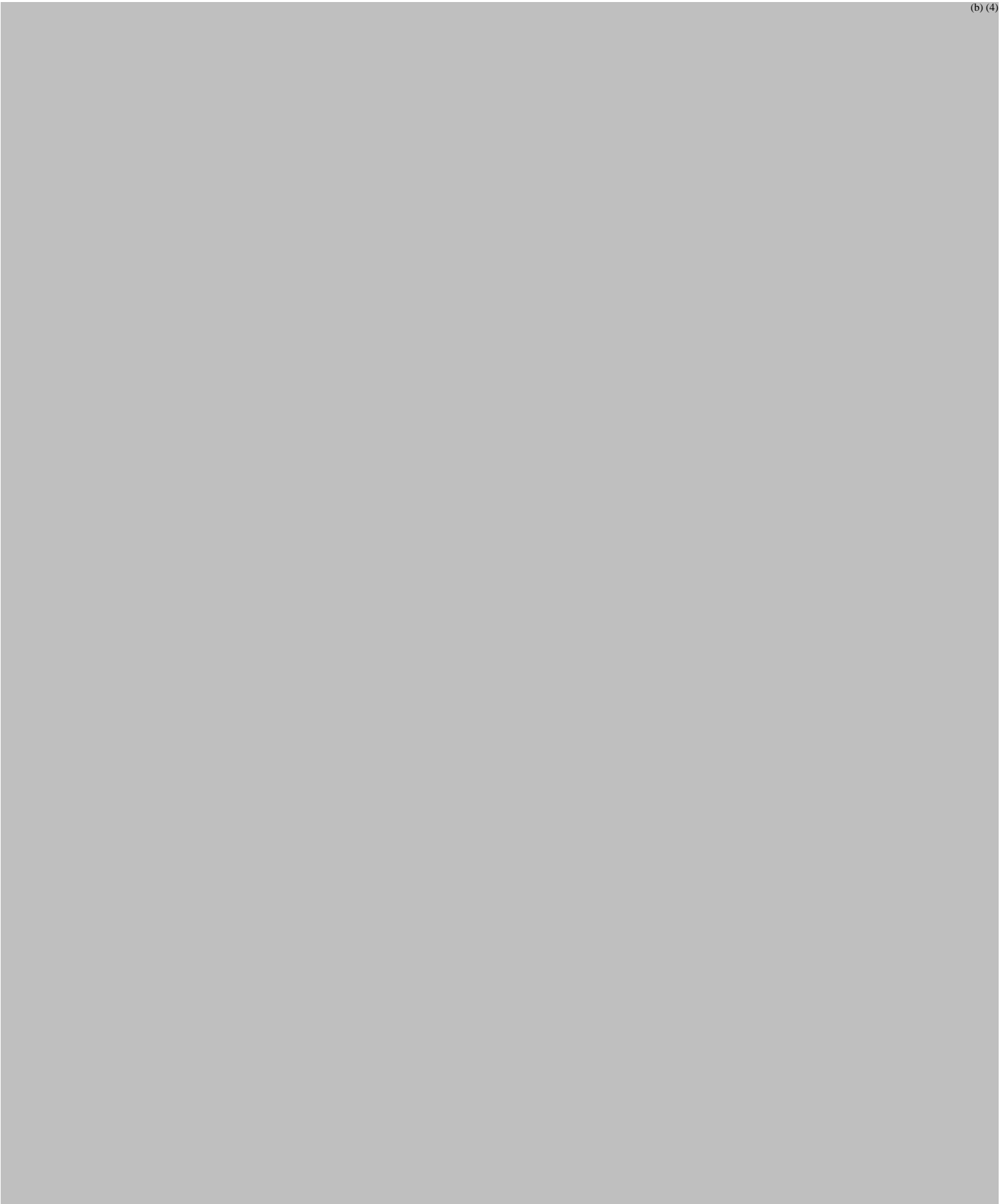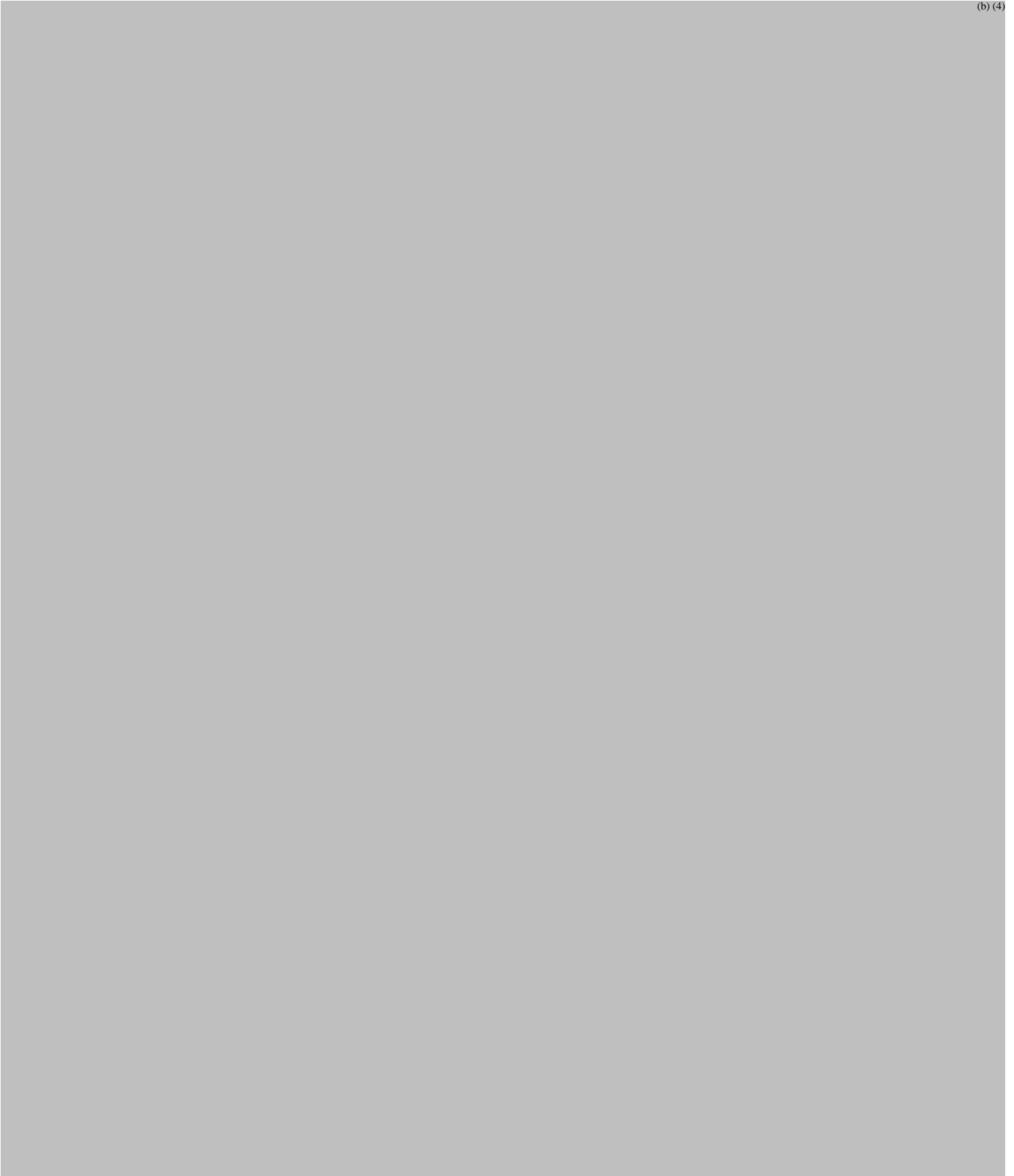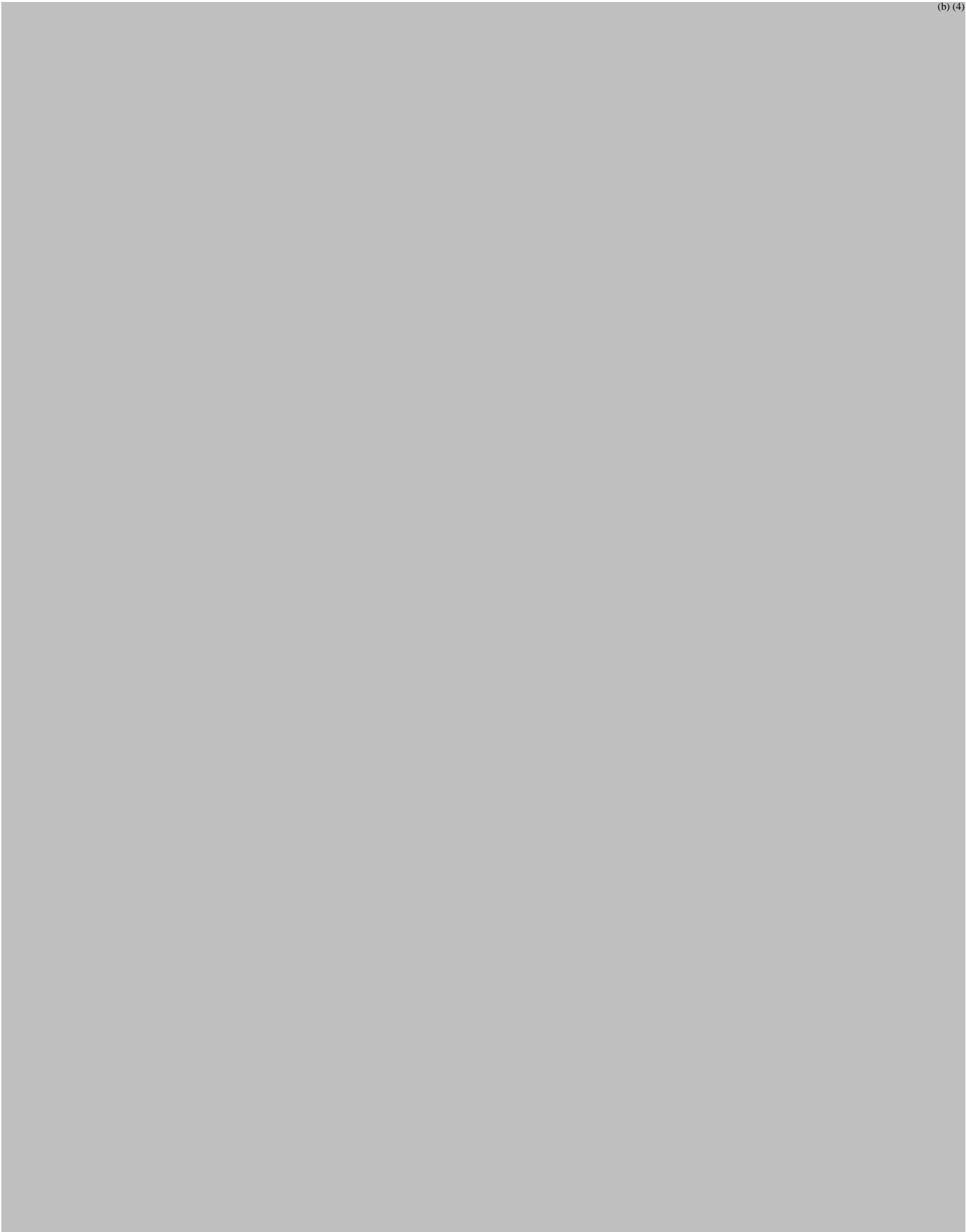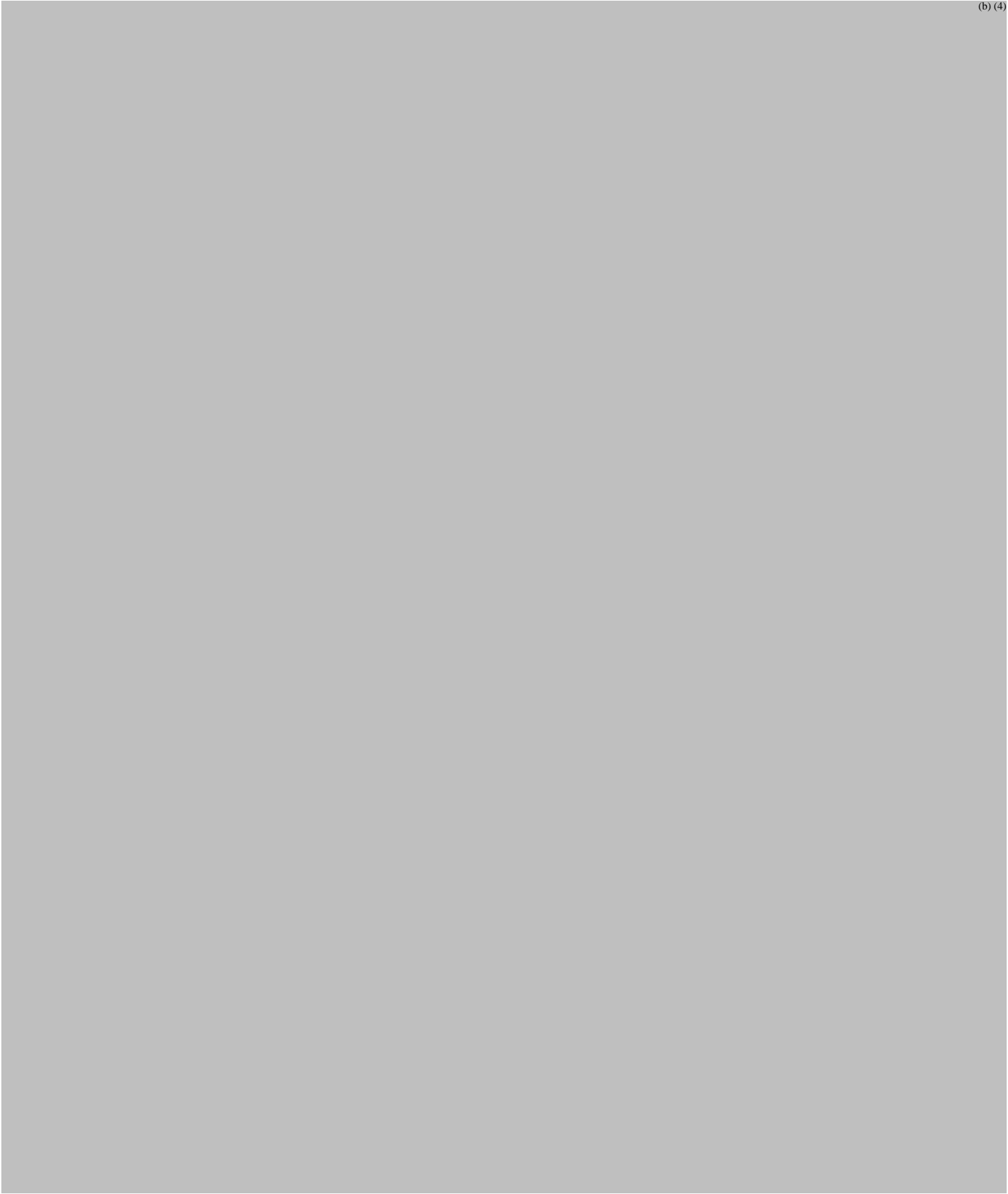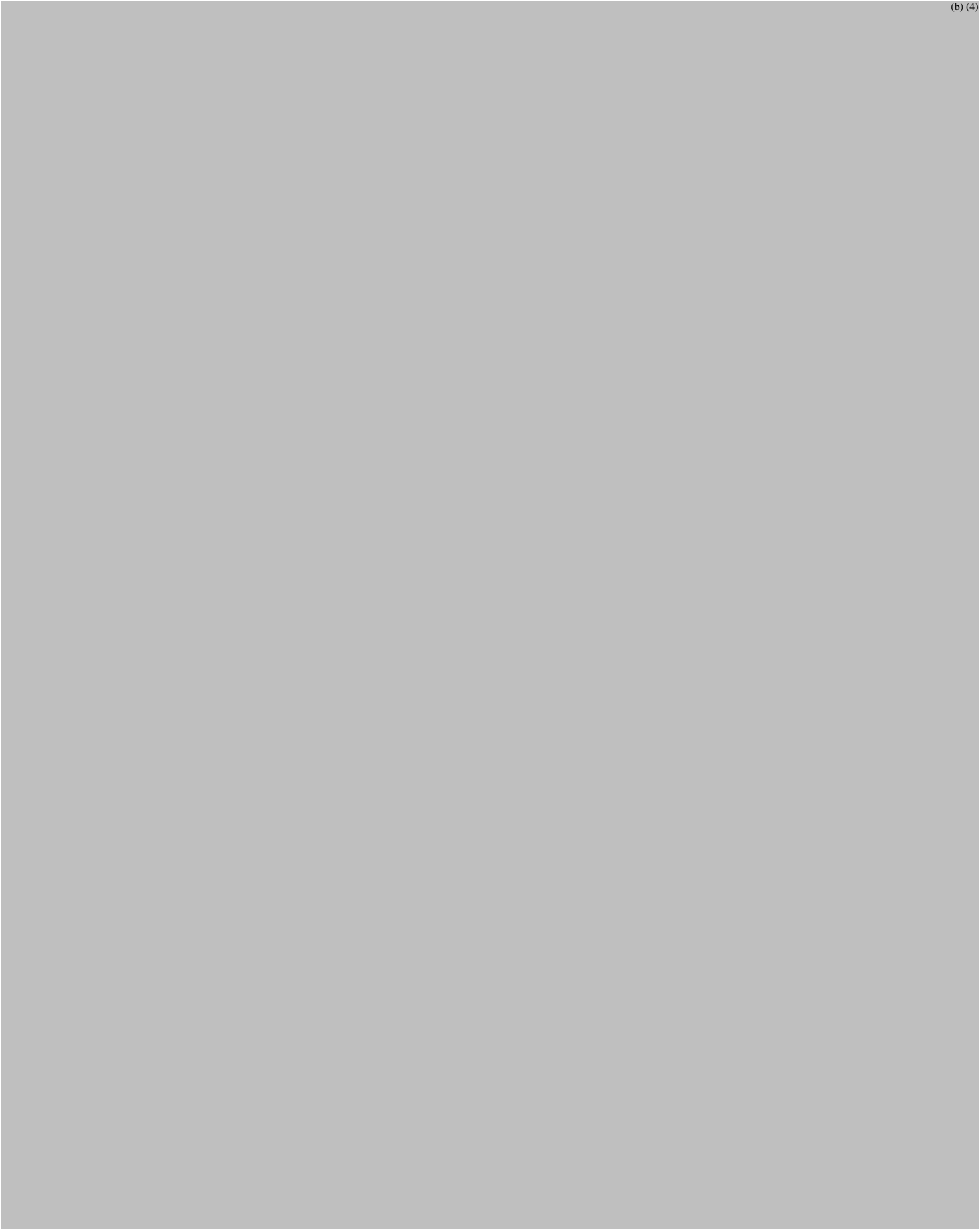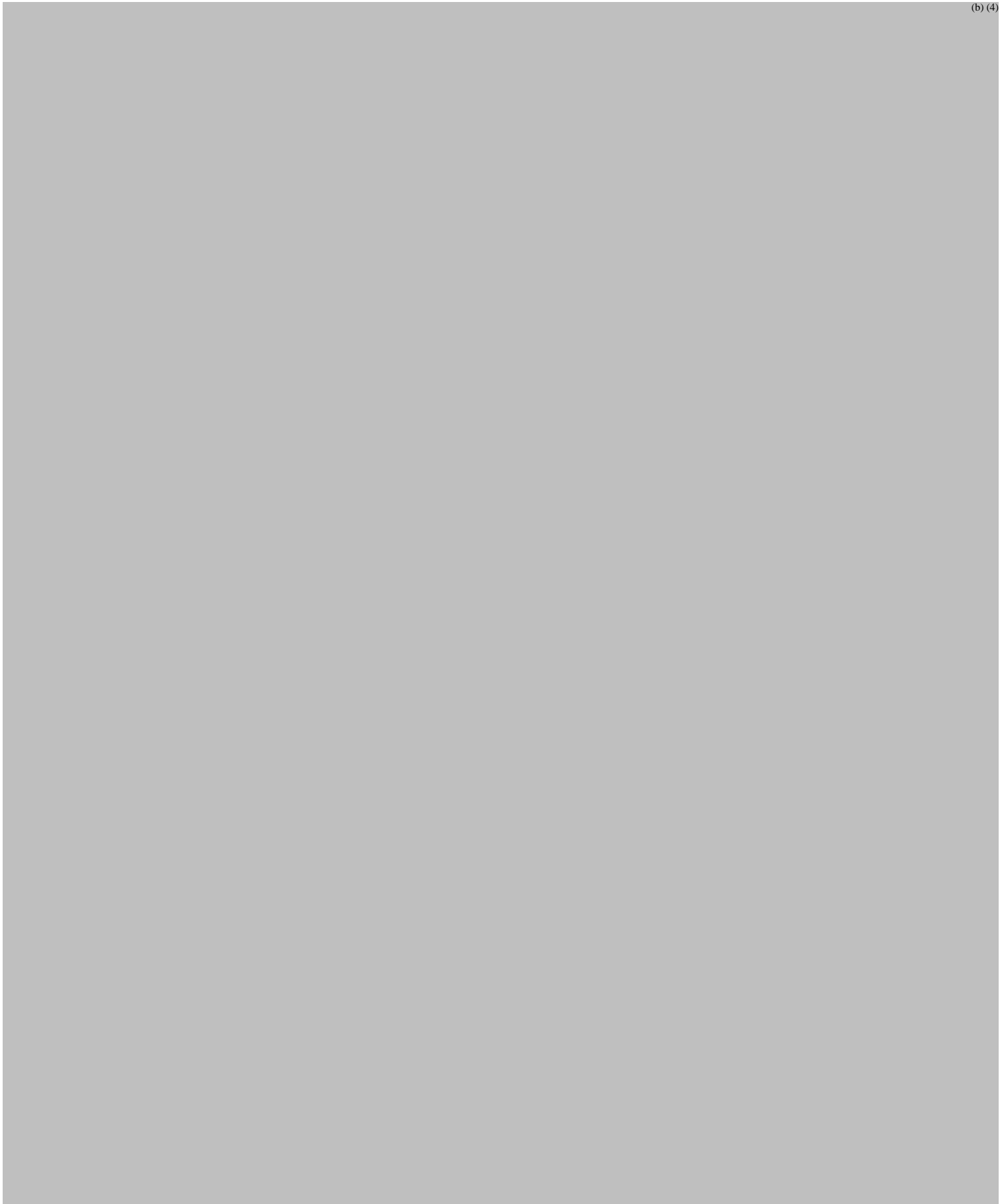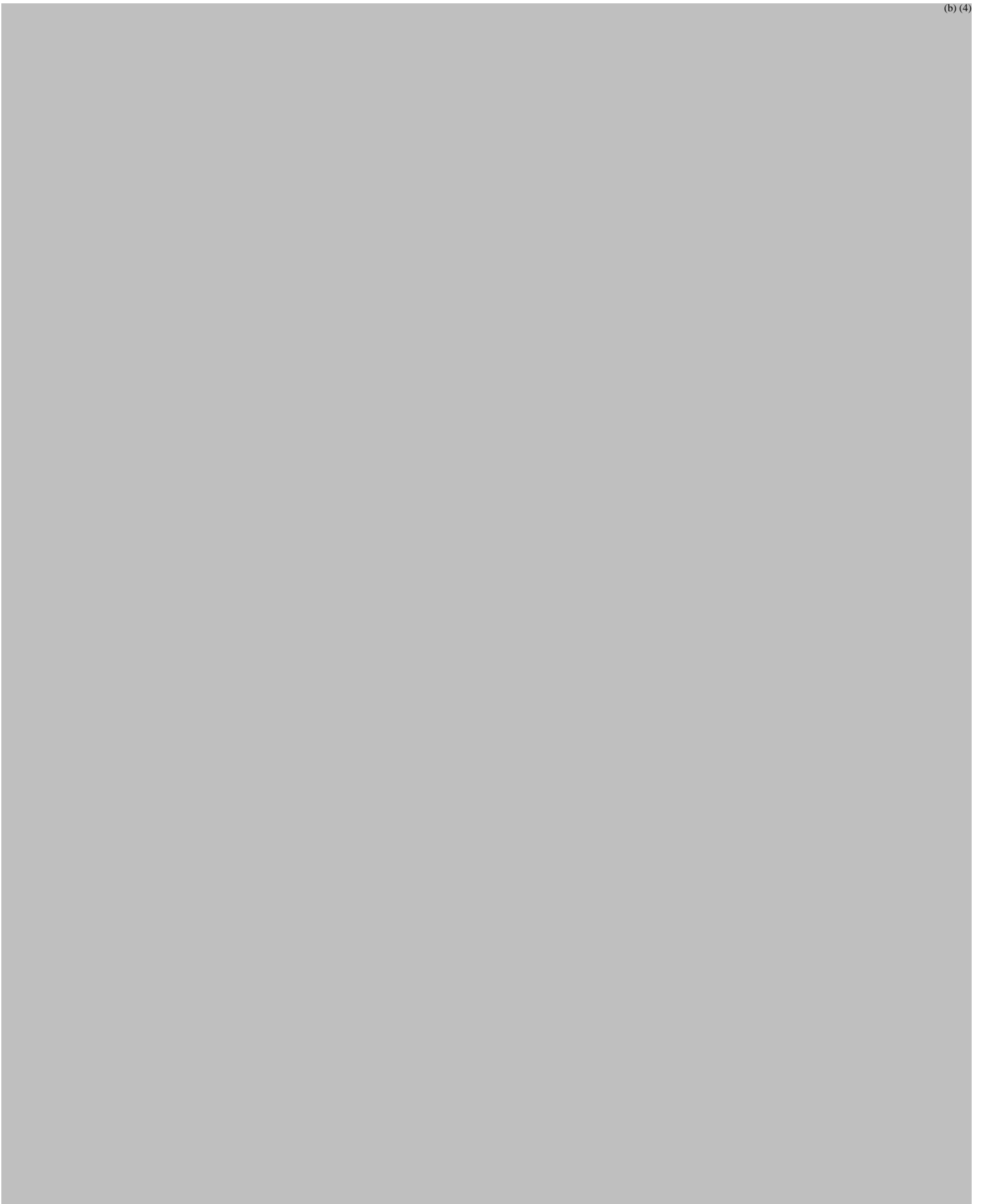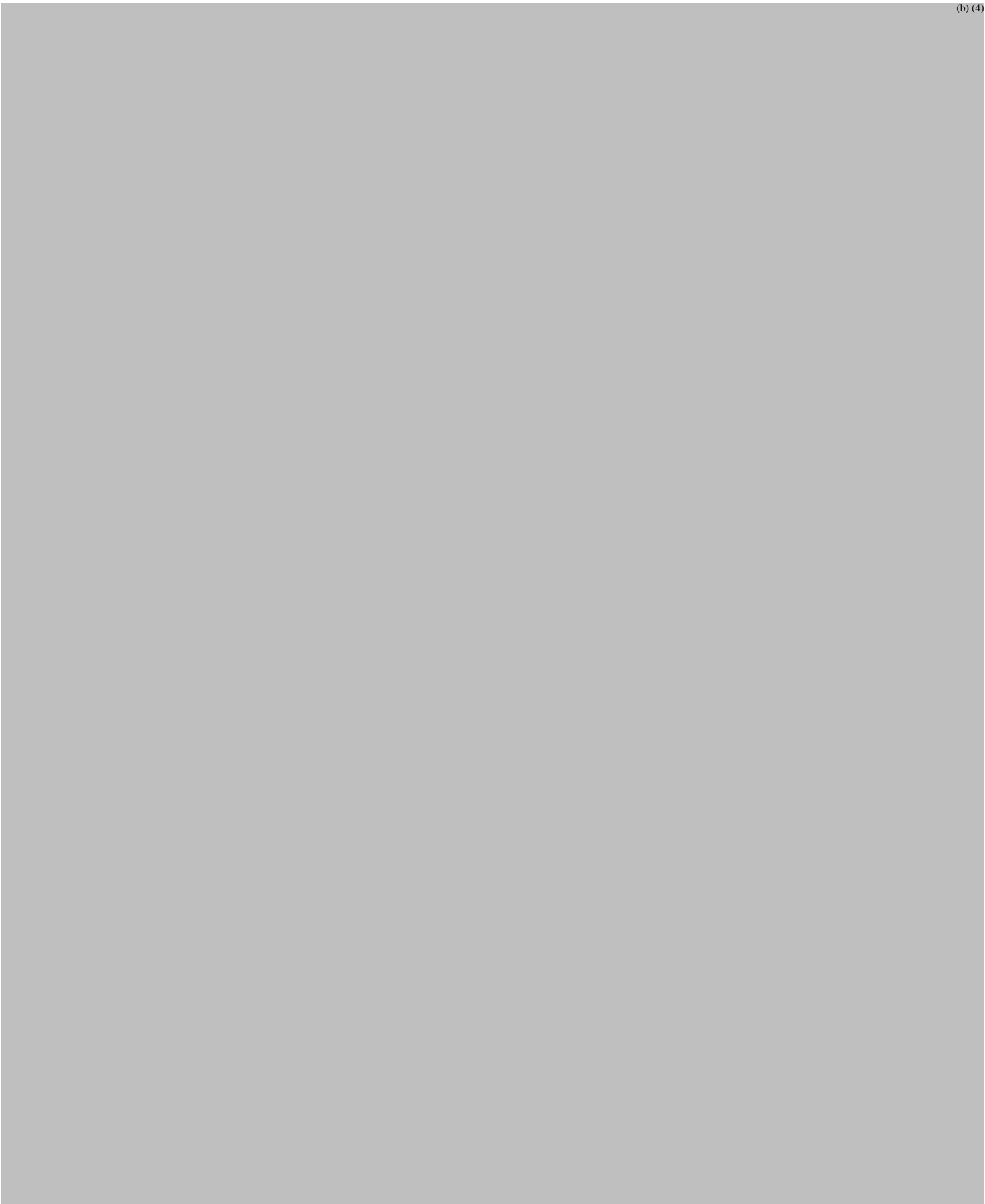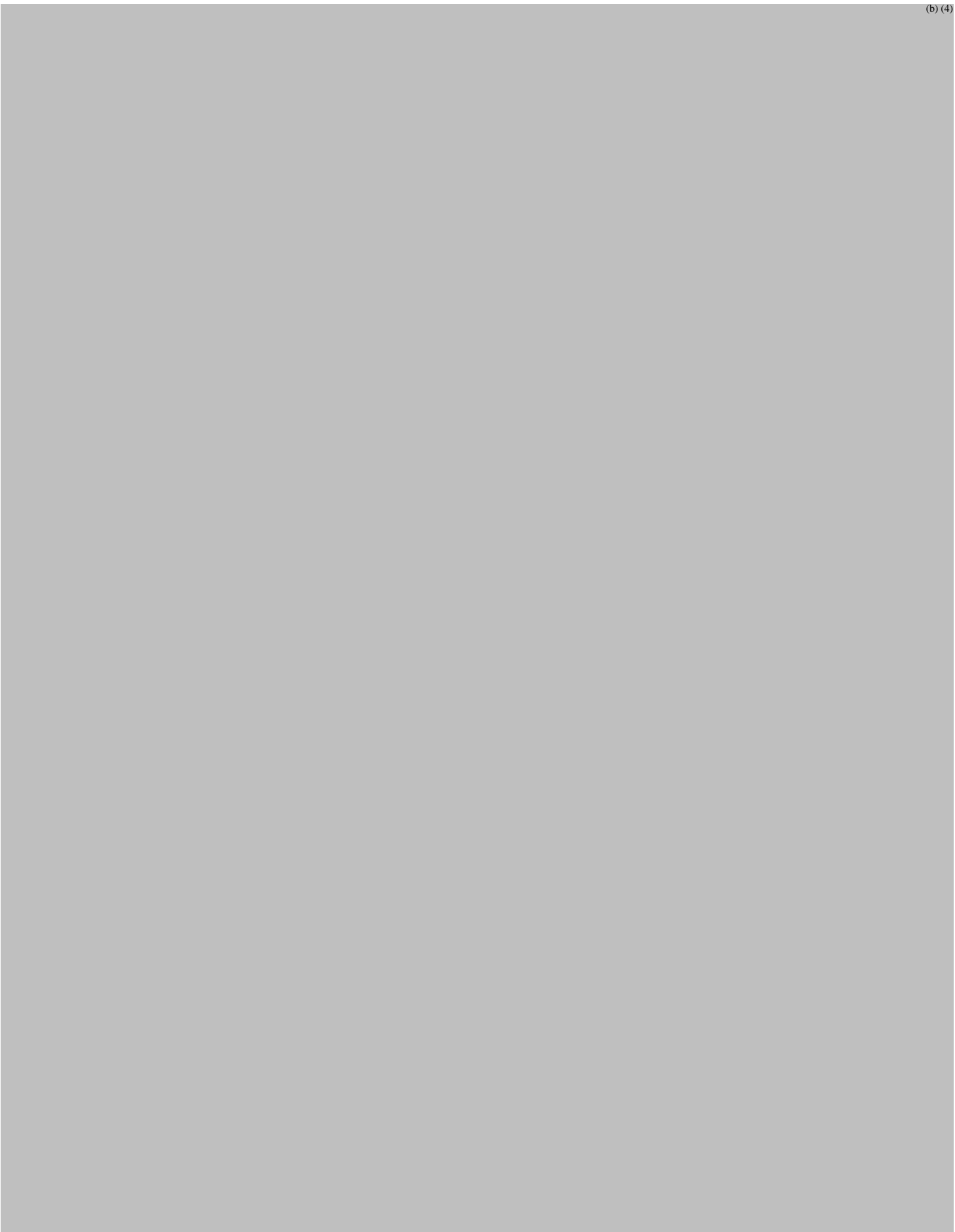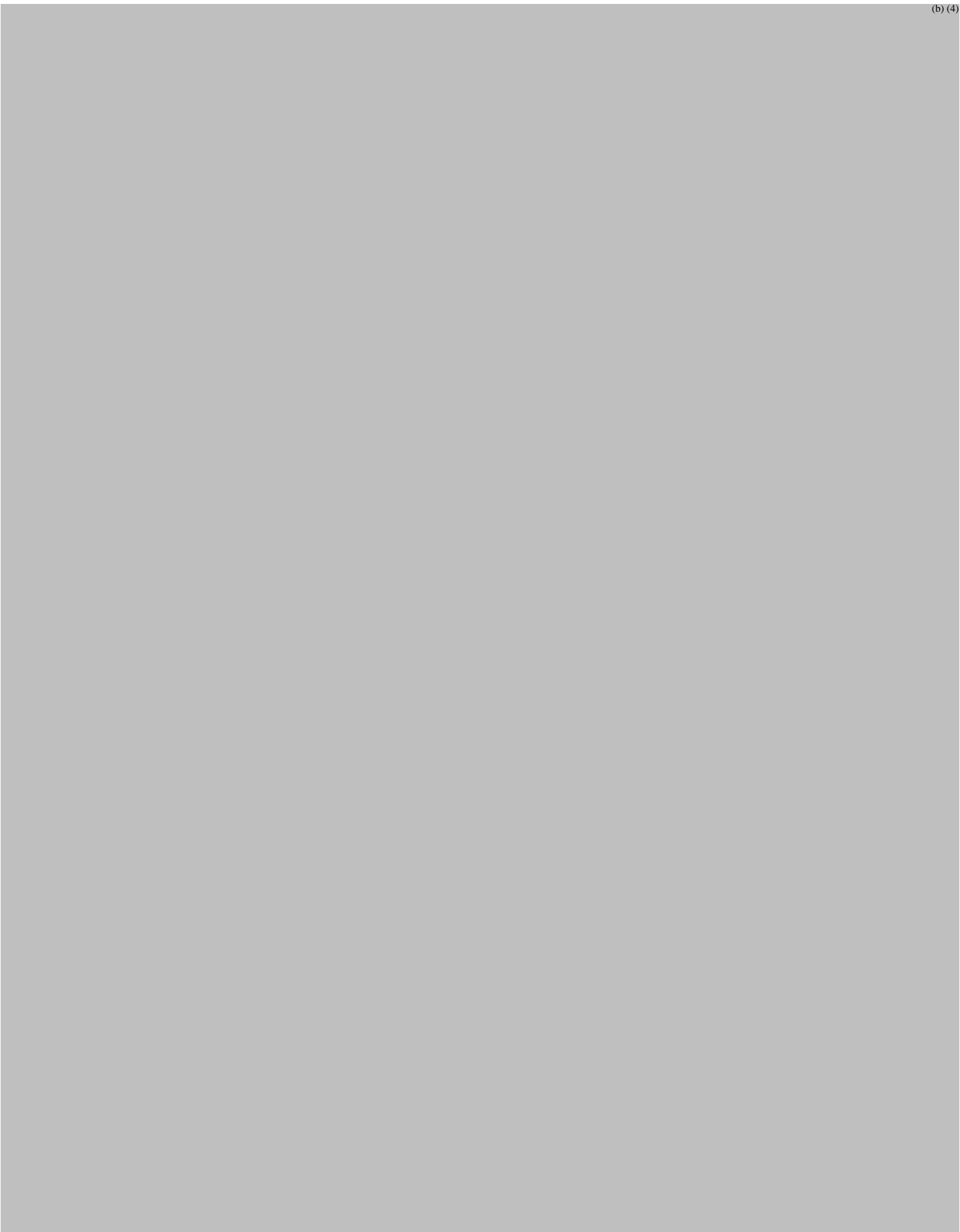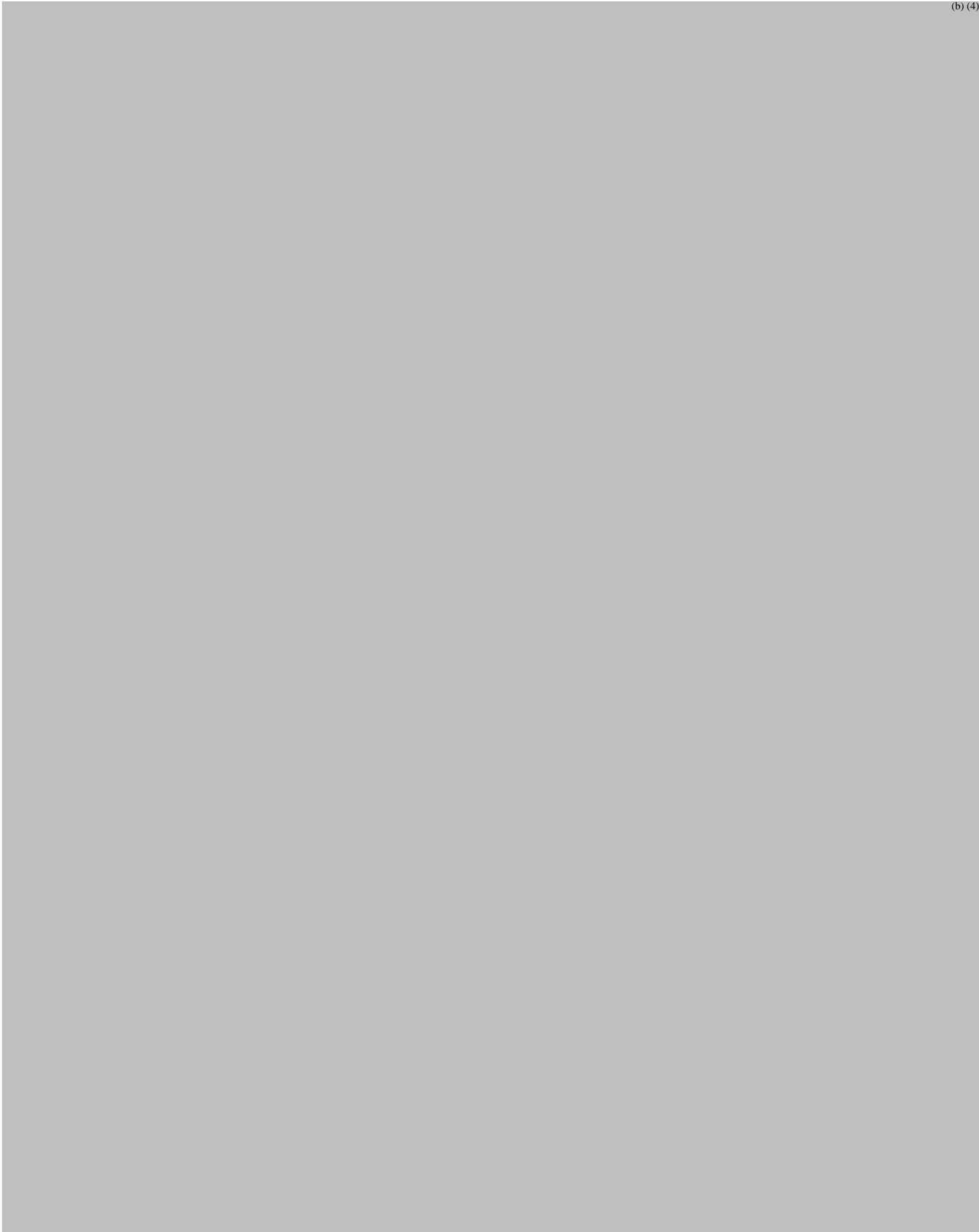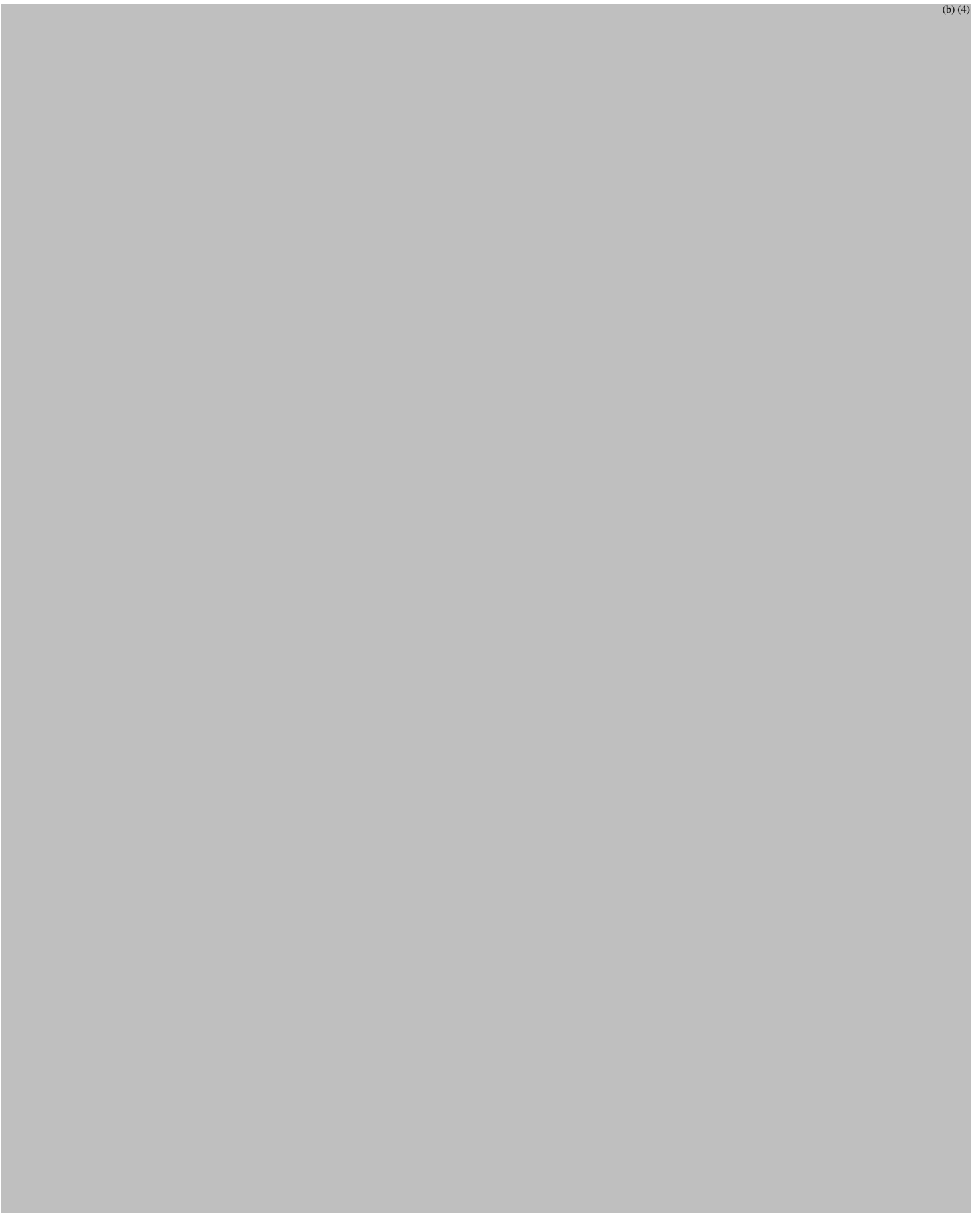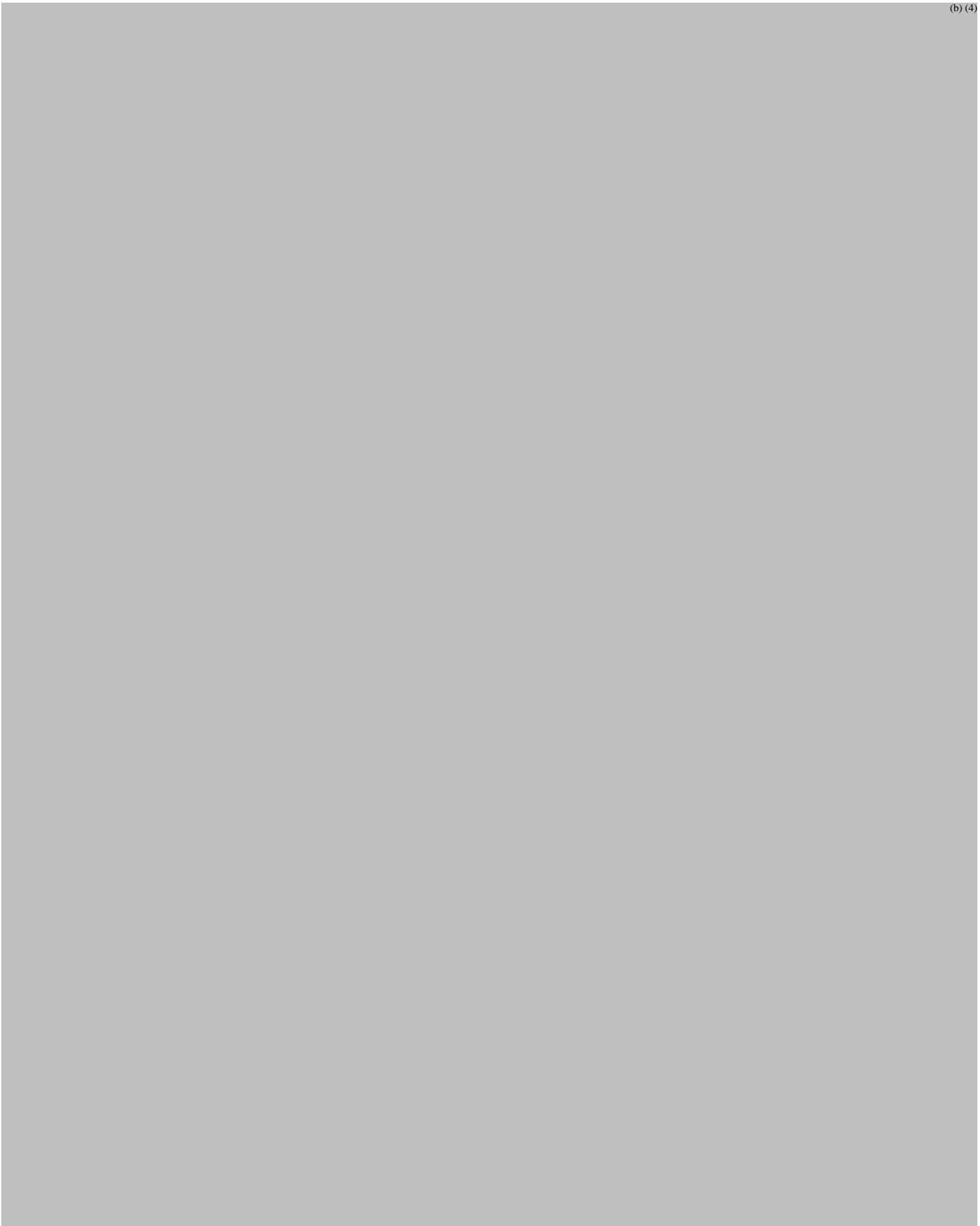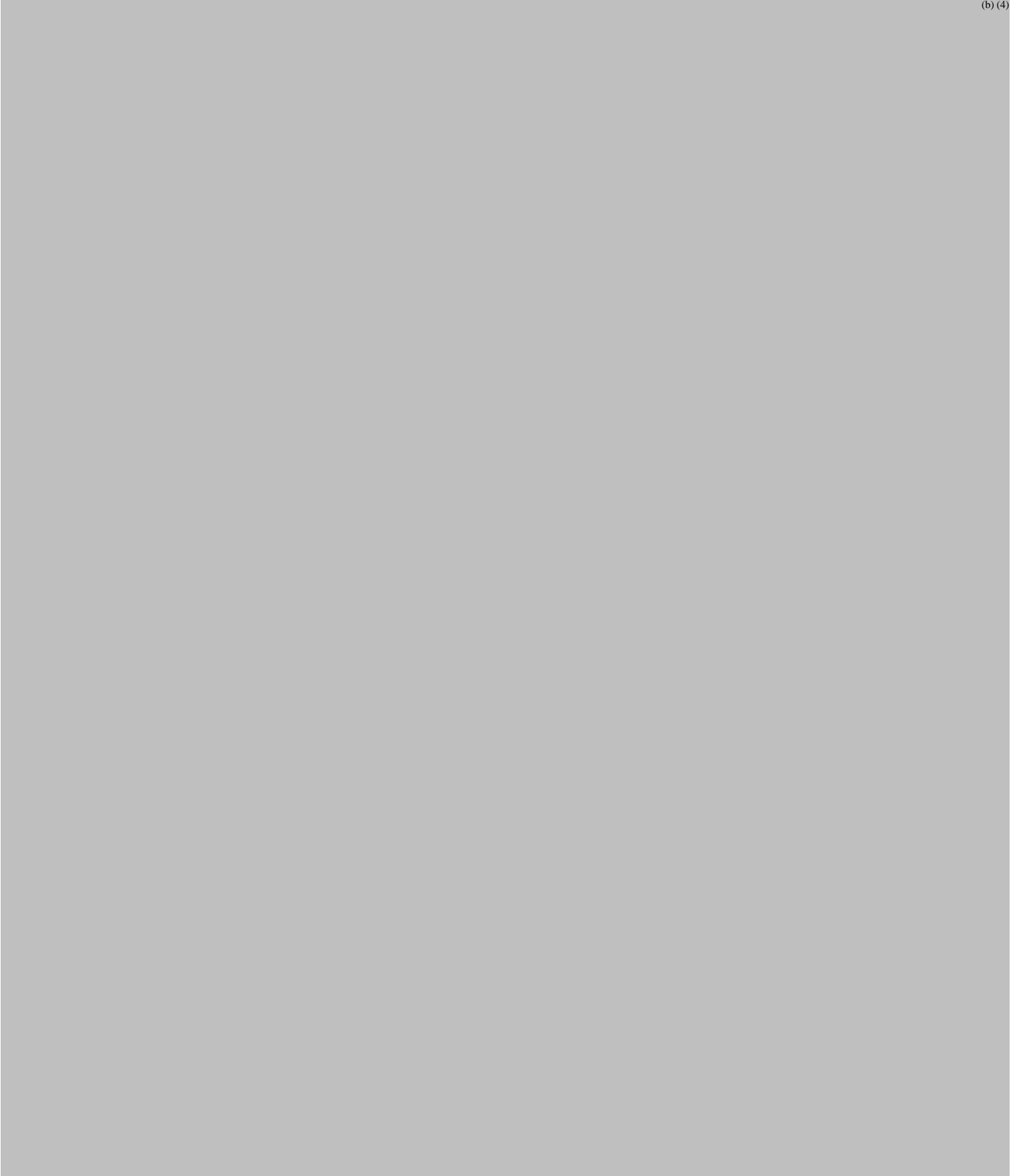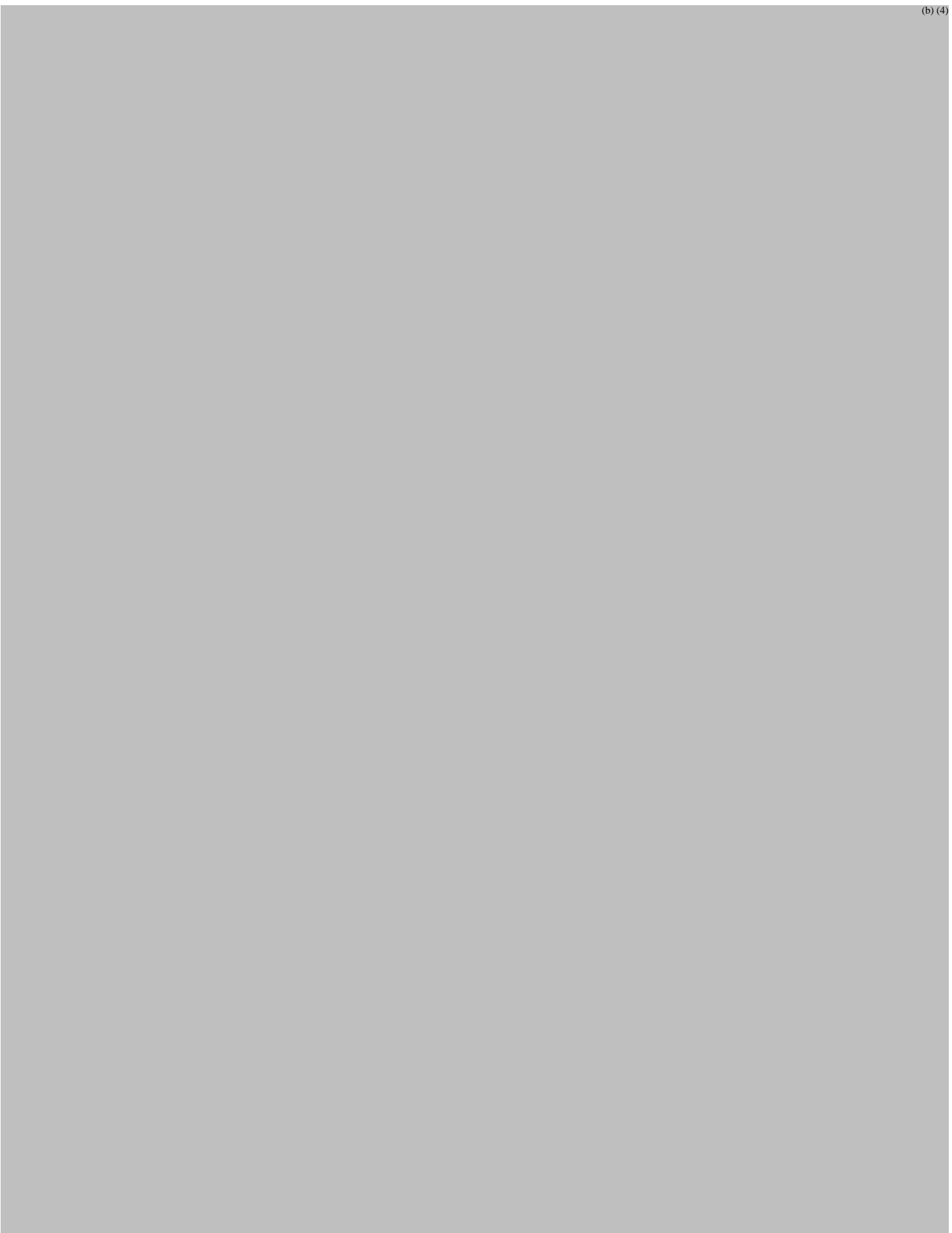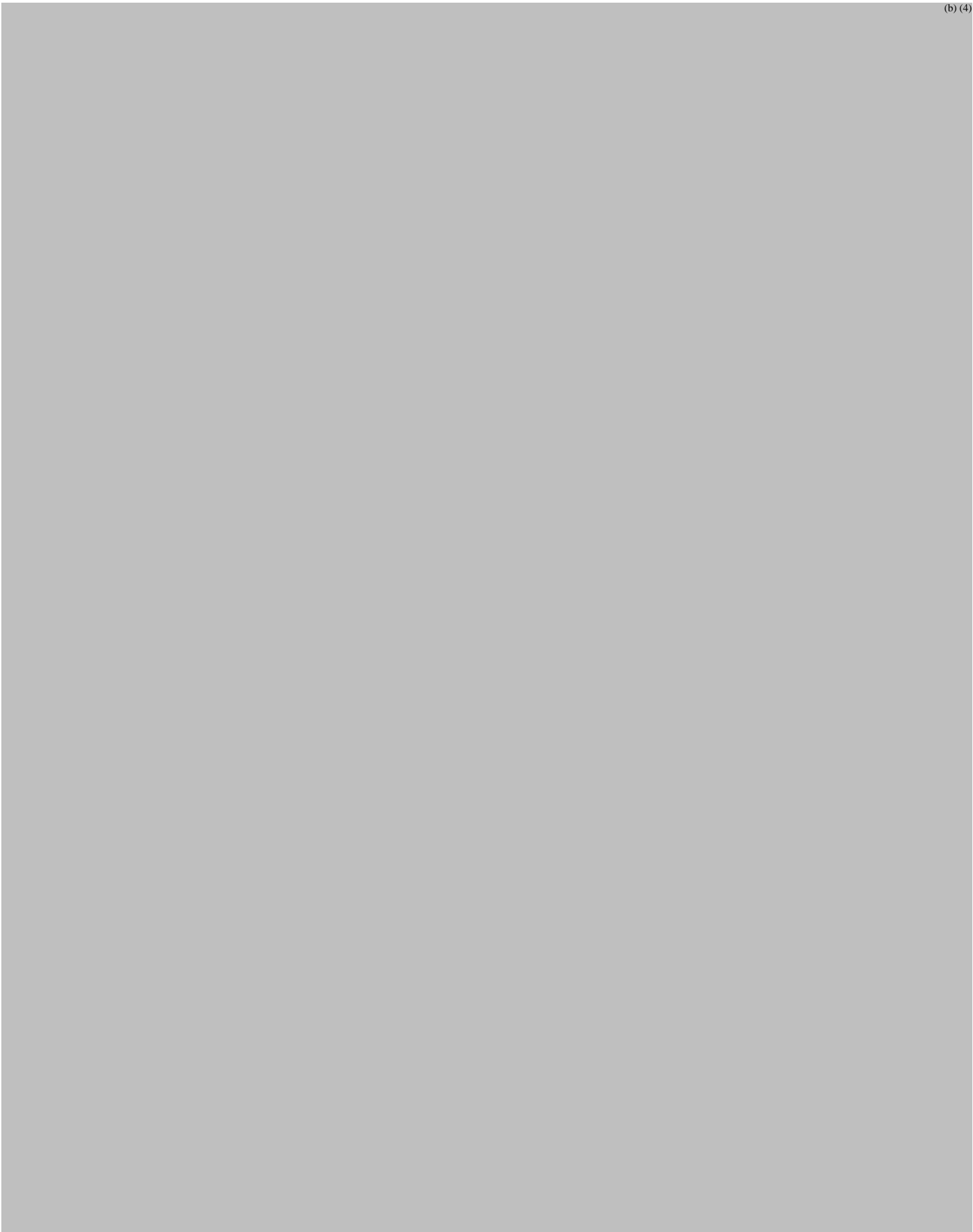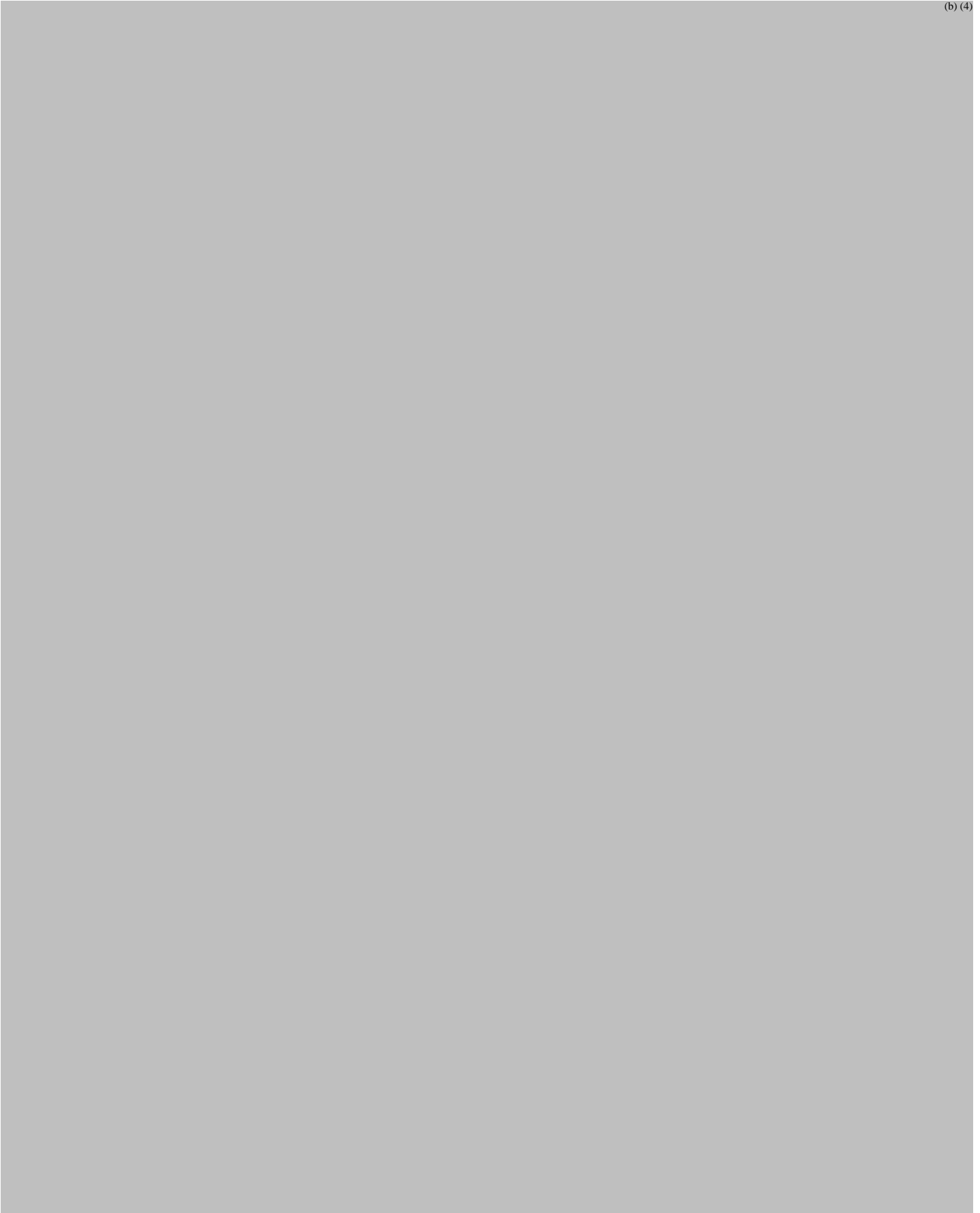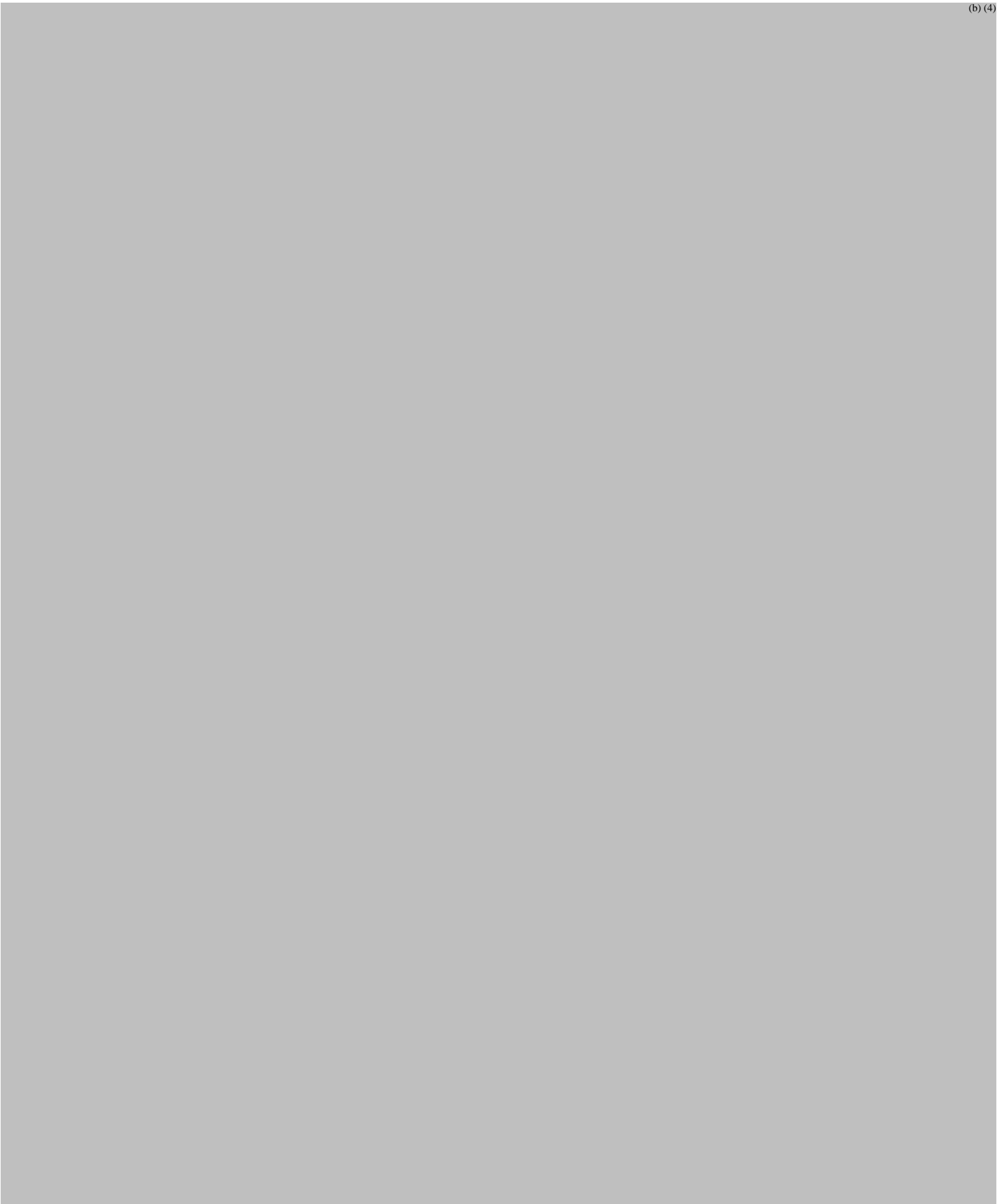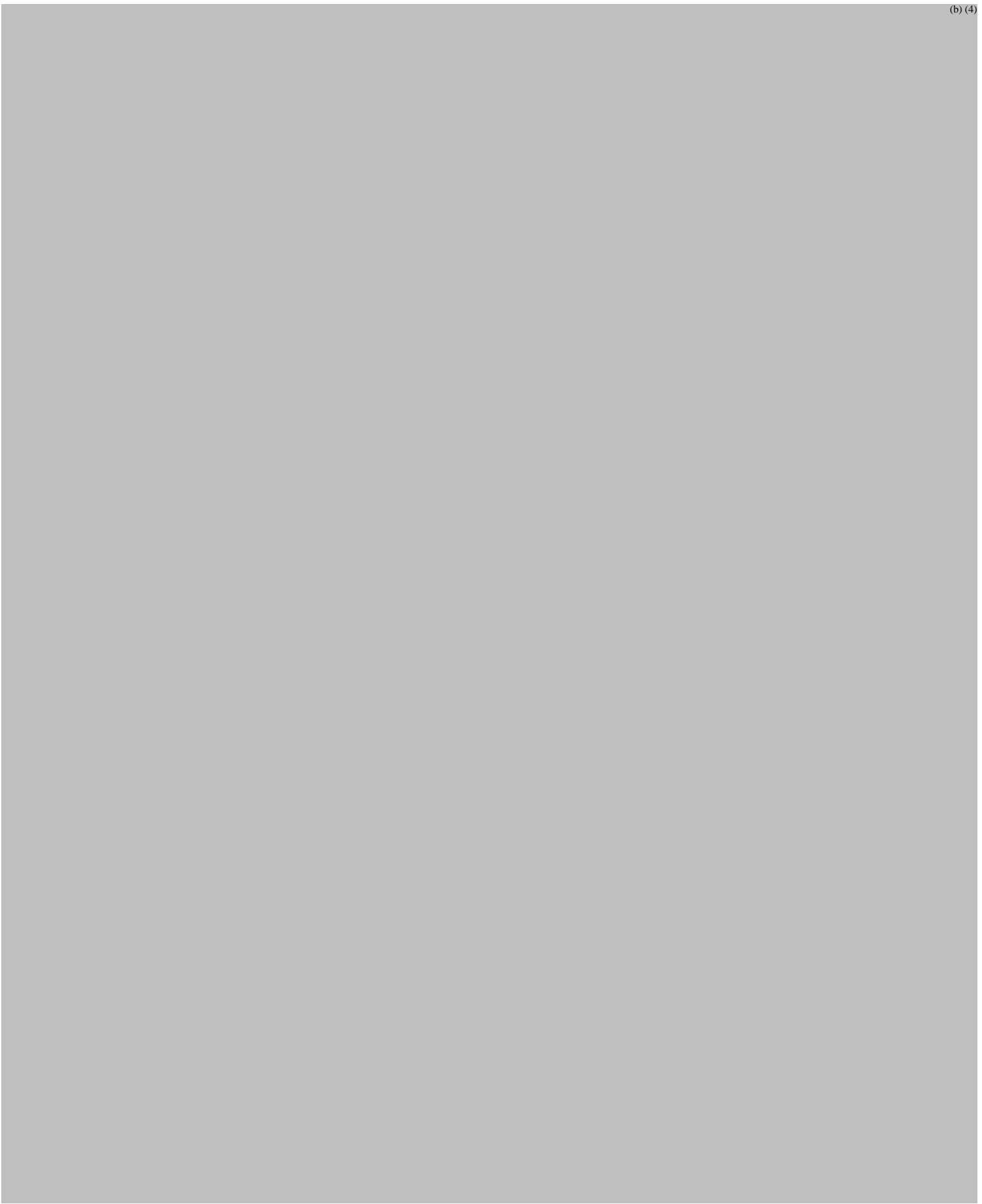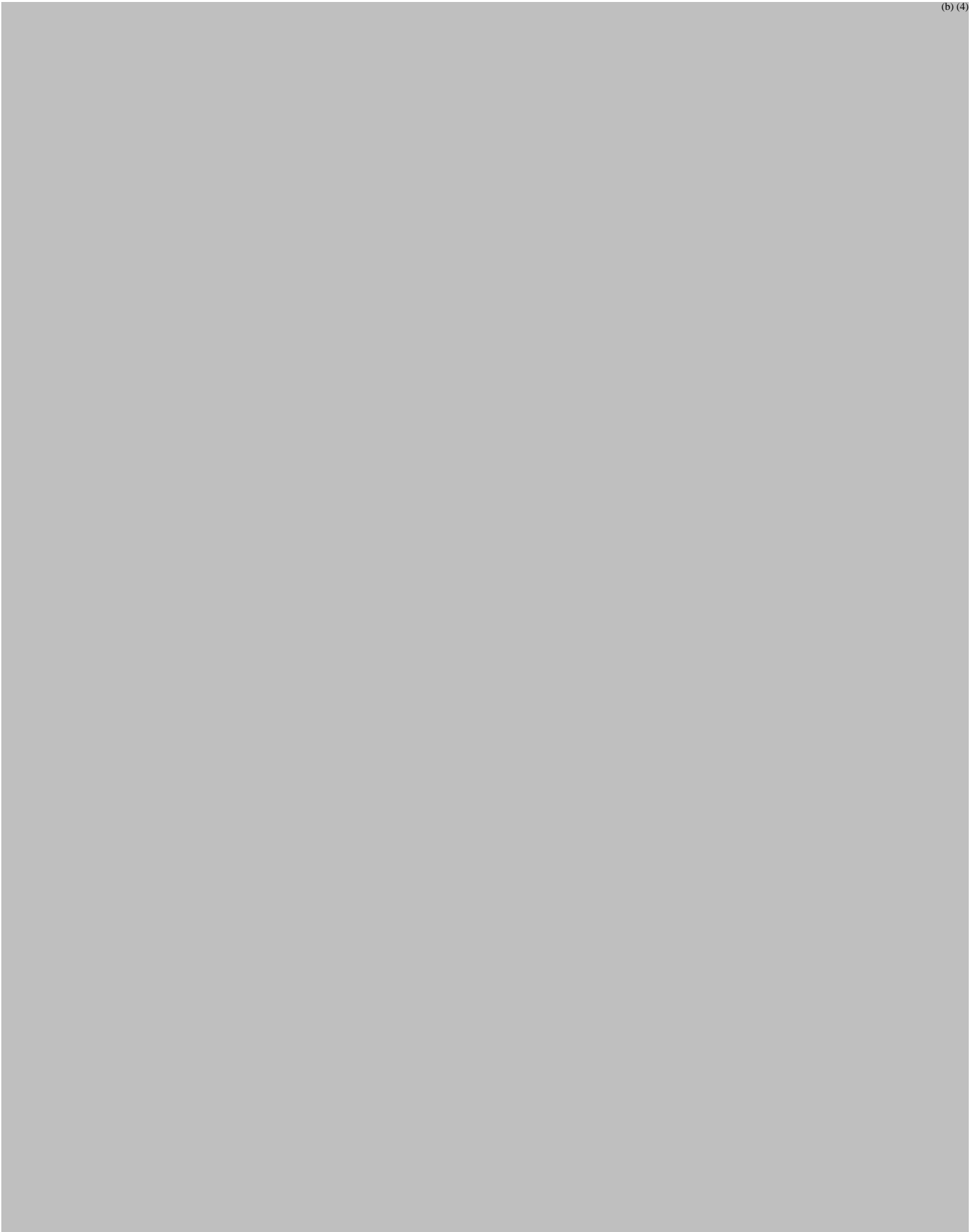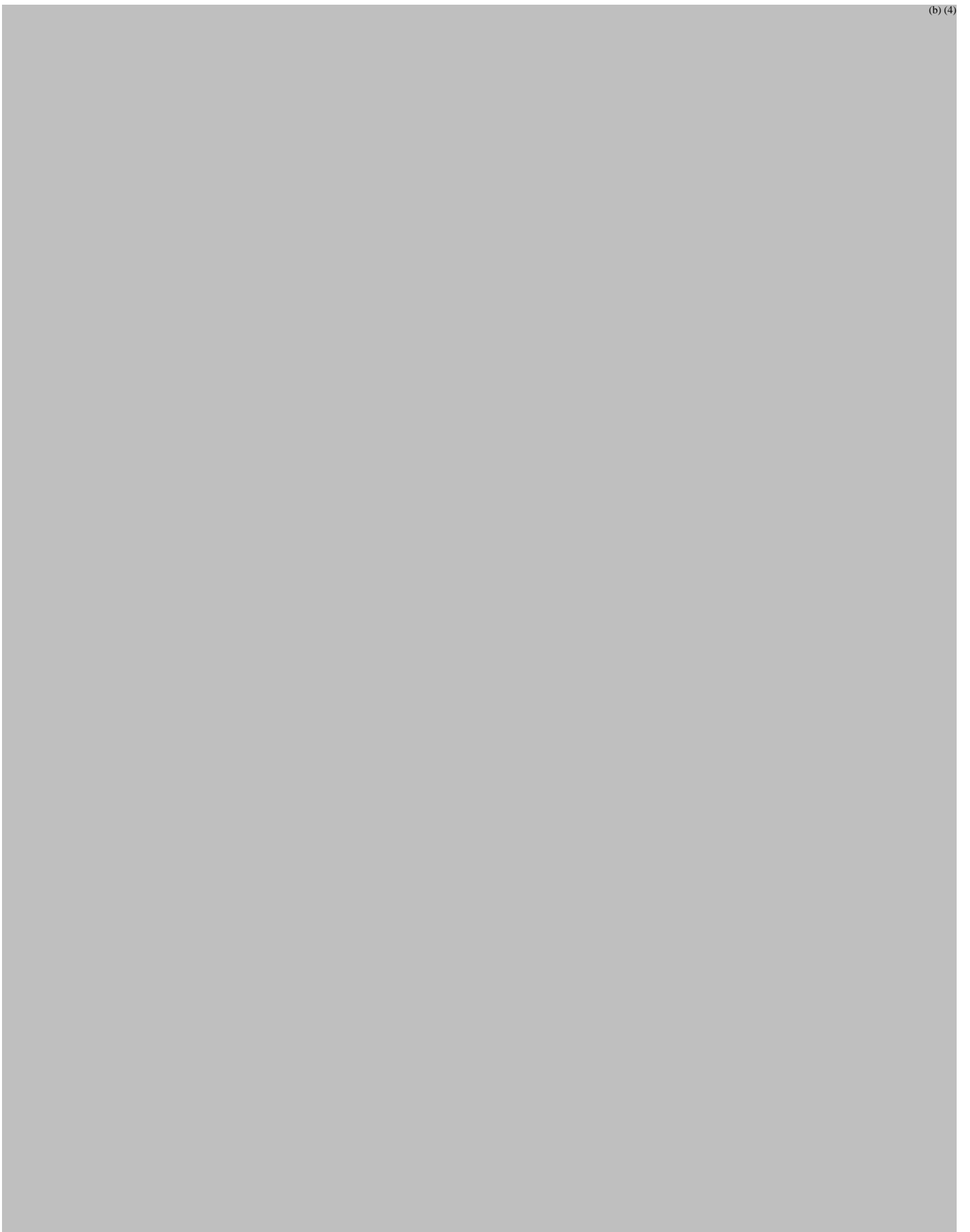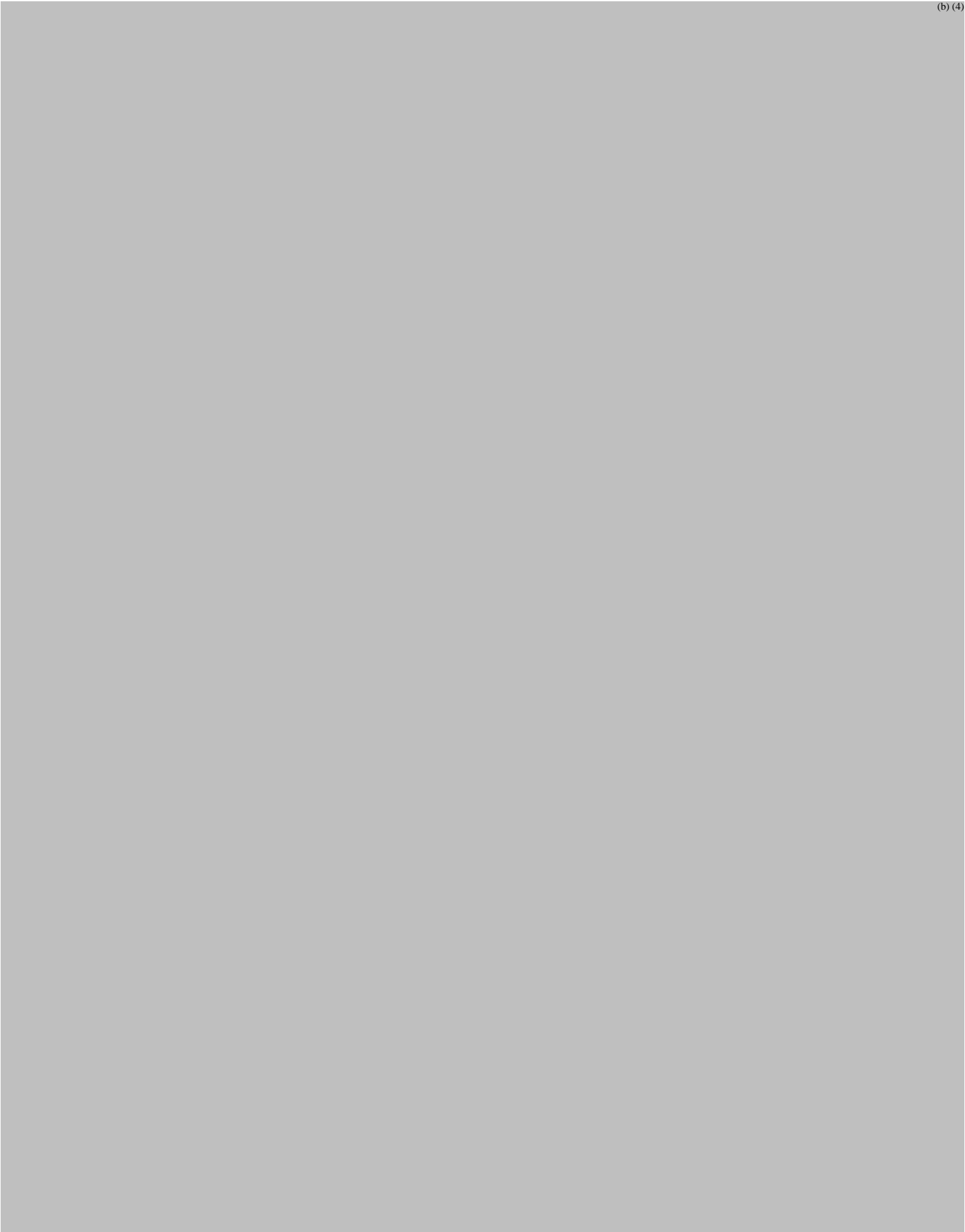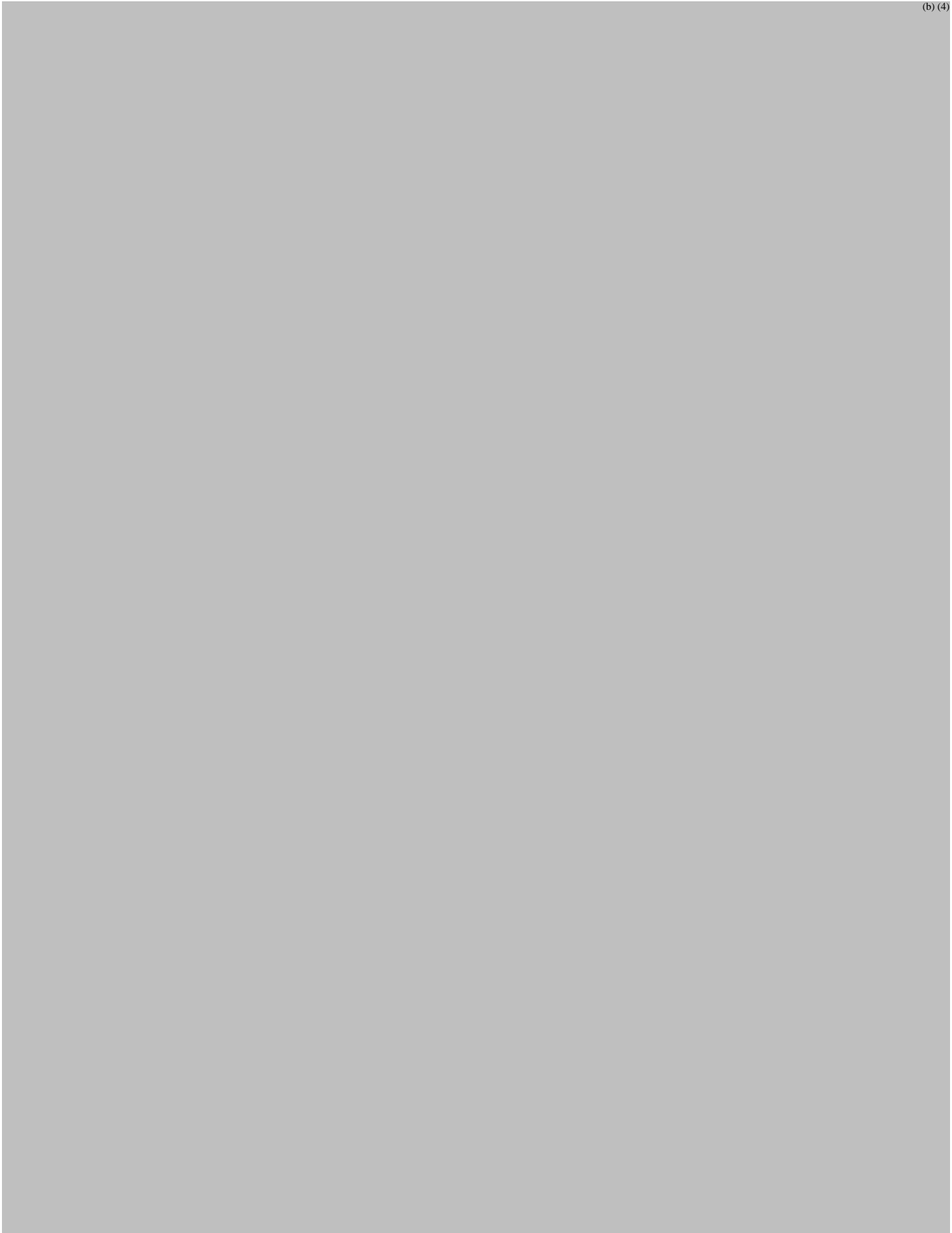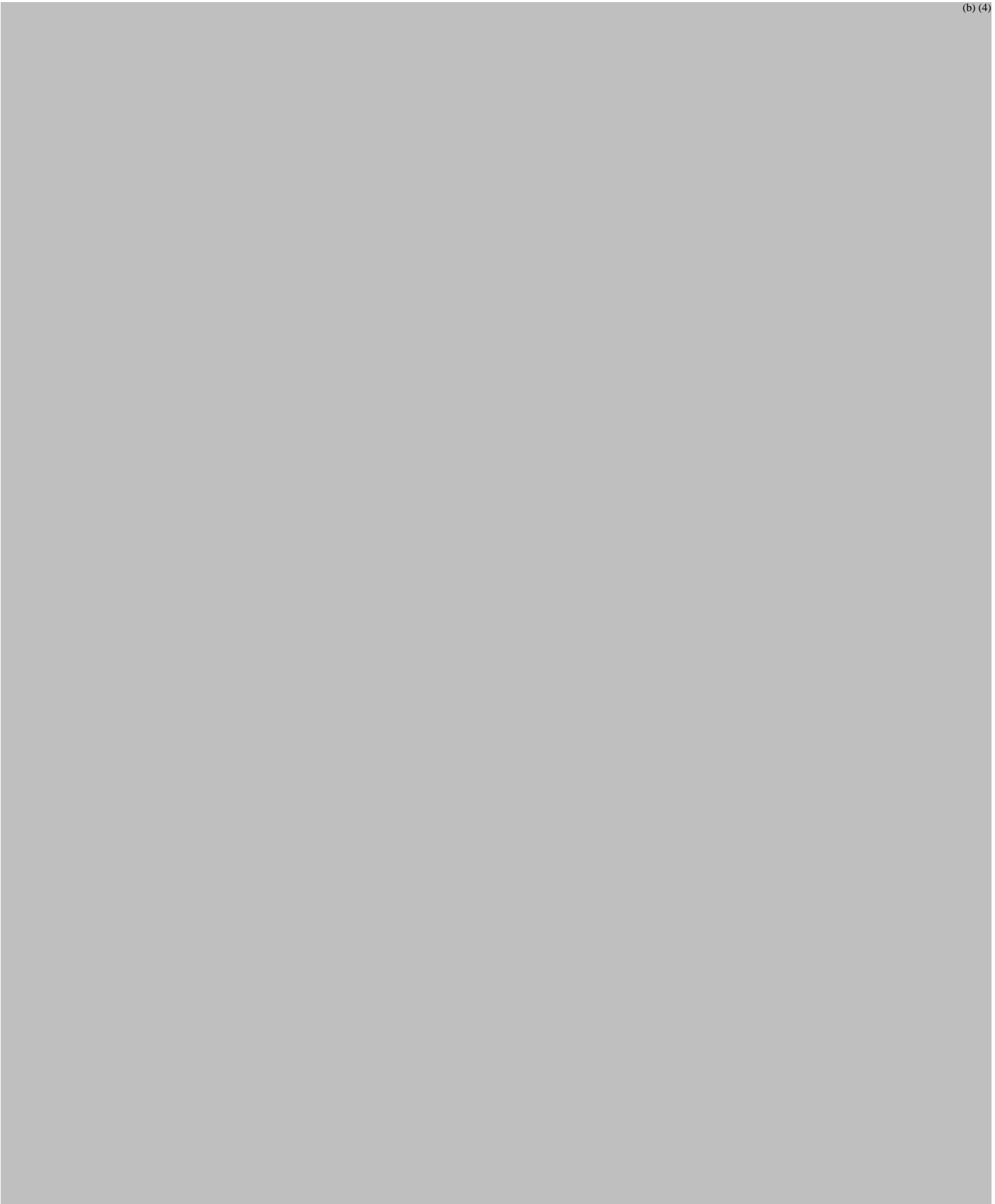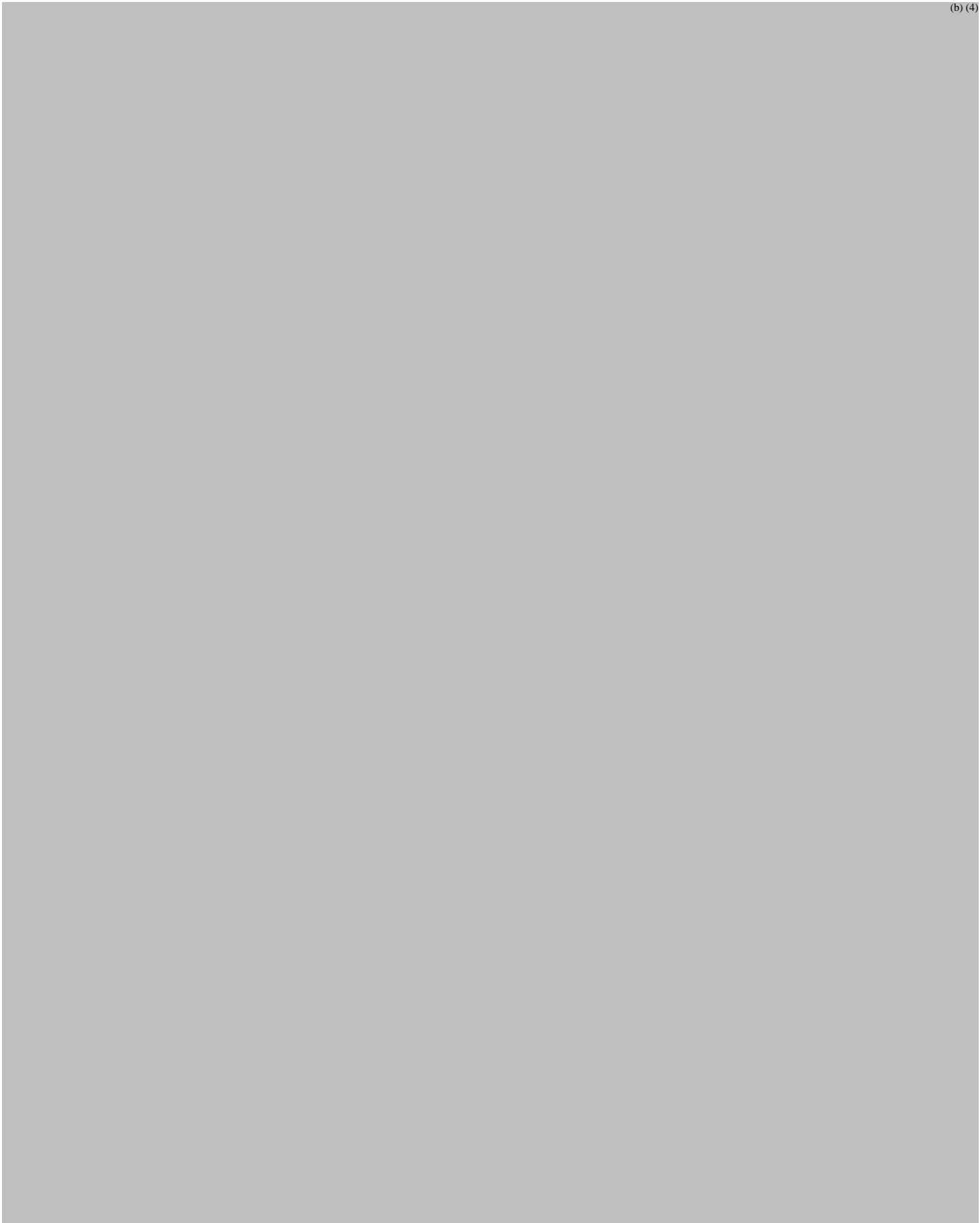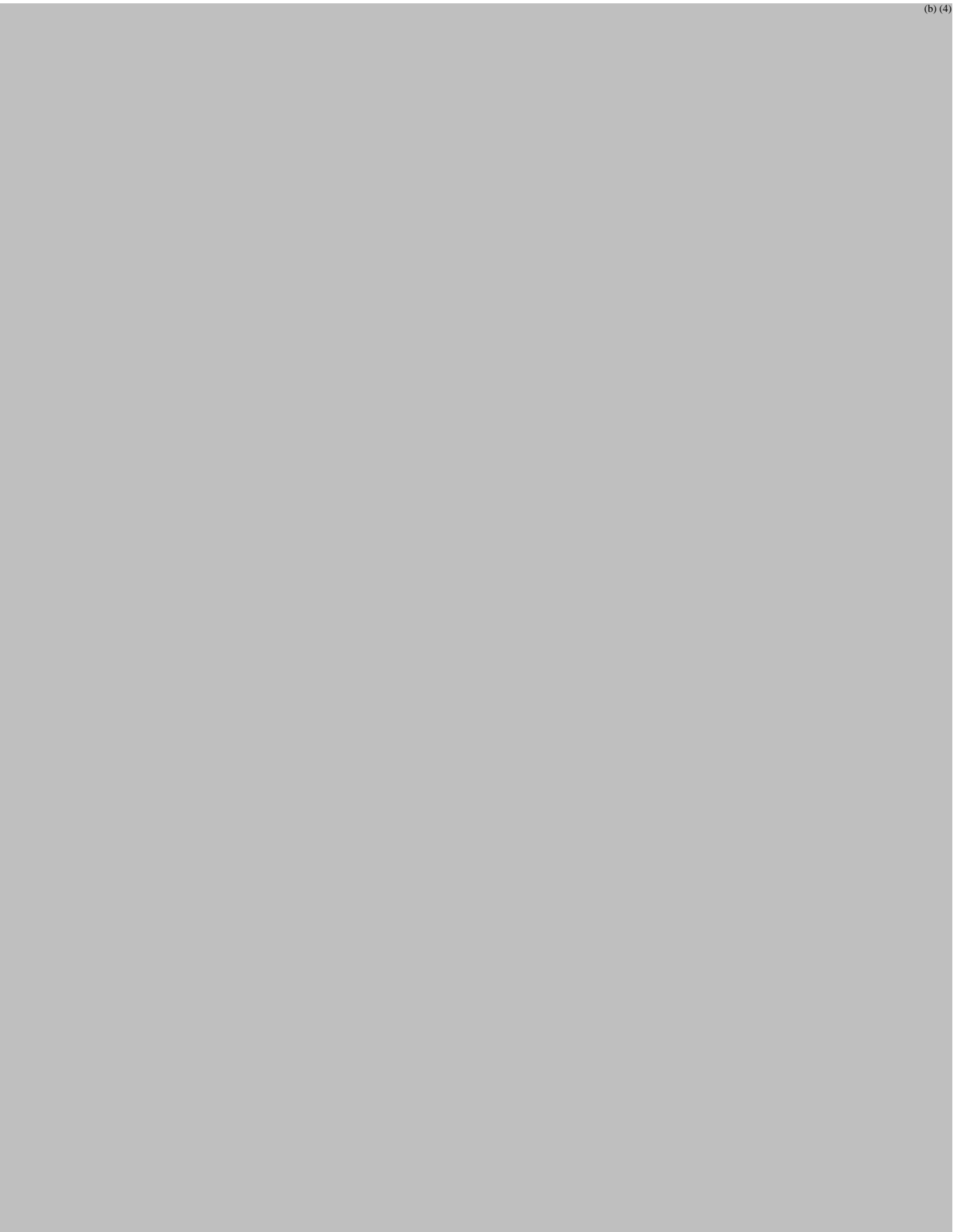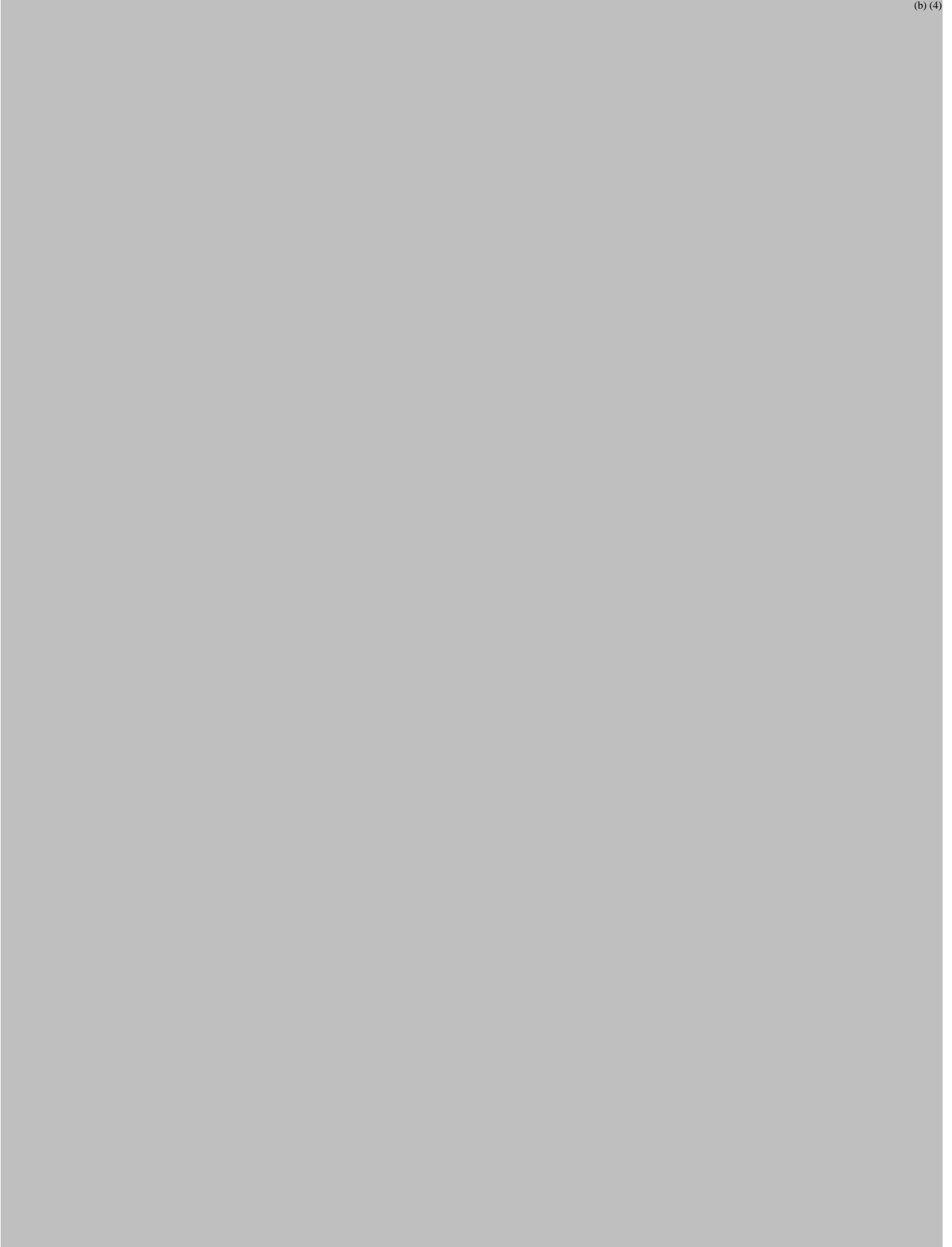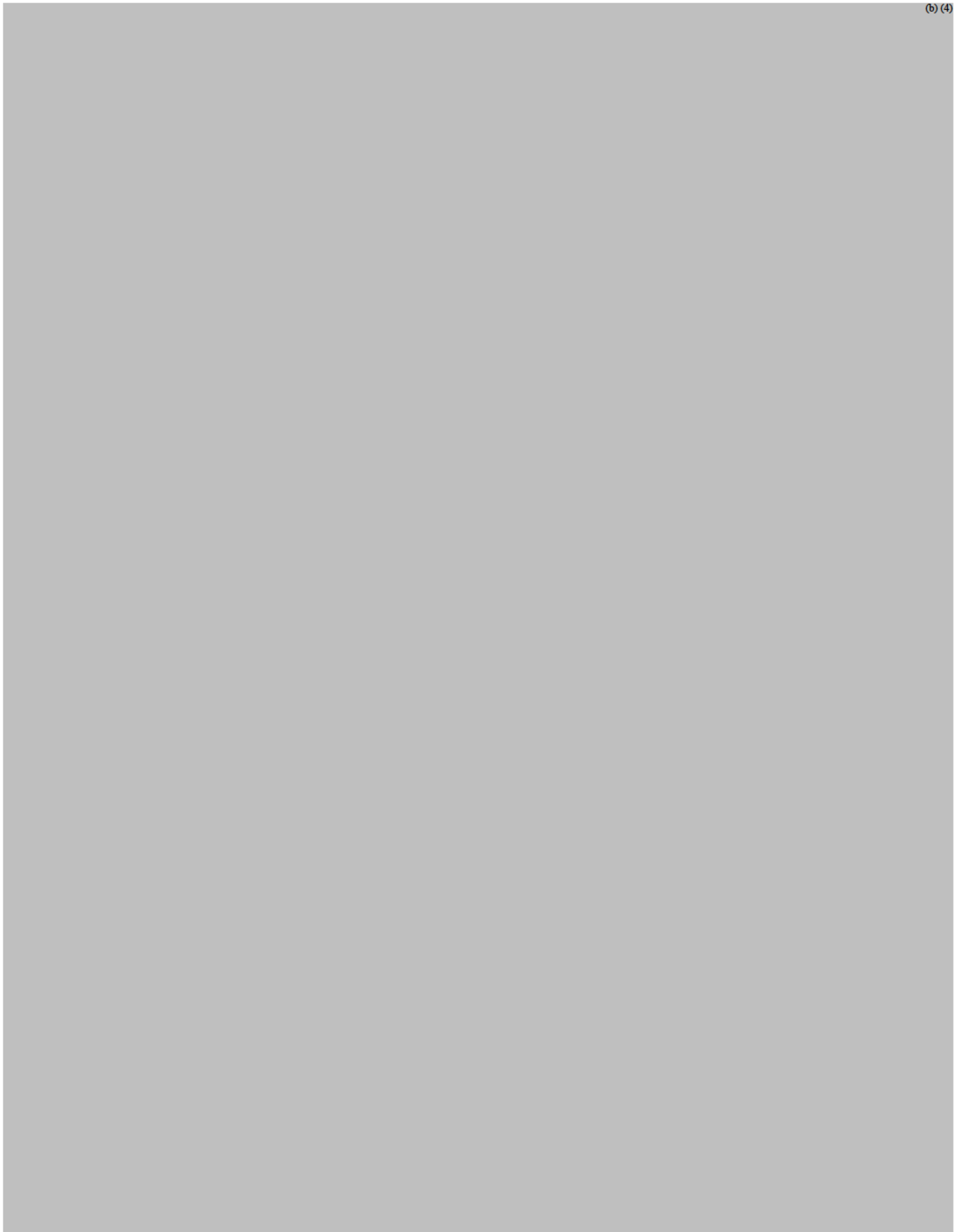
(b) (4)

(b) (4)

Appendix 003C: Supplementary Whole Genome Analysis Methods and Read Quality Metrics for *Clostridium beijerinckii* ASCUSDY20

The *Clostridium beijerinckii* ASCUSDY20 genomic DNA was extracted and sequenced as described in the main text of the dossier. This appendix contains details about the assembly methods used, the protocol for NexteraXT library preparation, FastQC and NanoStat quality metrics for the Illumina and Oxford Nanopore reads respectively, metrics generated by Quast for the completed assembly, and a visualization of the assembly graph generated by Bandage.

Assembly Pipeline in Detail

NexteraXT Protocol as Provided by the Manufacturer

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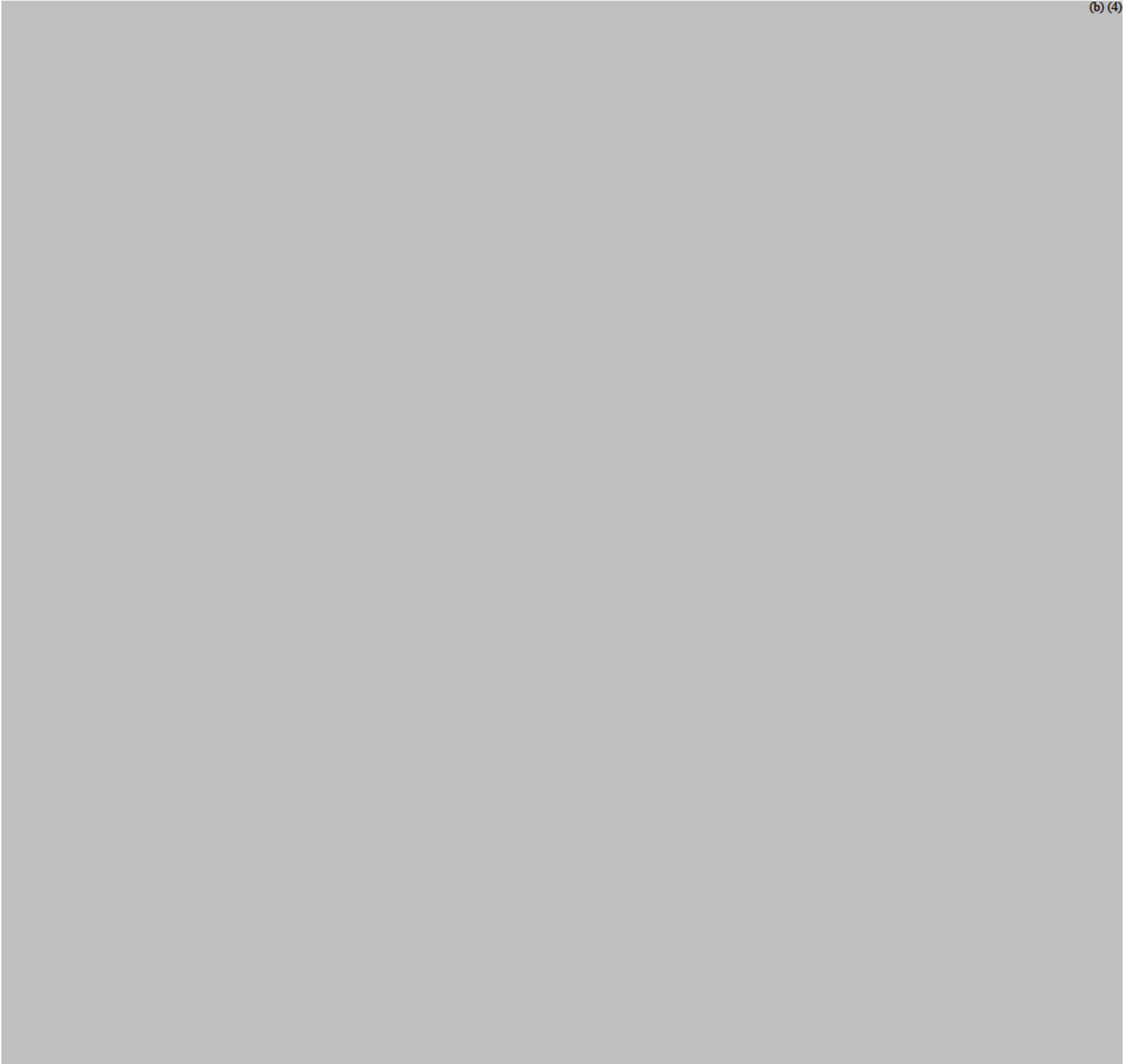
Quality Metrics of Illumina Reads as Generated by FastQC

(b) (4)

Read distribution as related to quality score

(b) (4)

Metrics for *Clostridium beijerinckii* ASCUSDY20 Oxford Nanopore reads as generated by NanoStat.

General Summary

(b) (4)

Number, Percentage, and Megabases of Reads Above Quality Cutoffs

(b) (4)

Assembly Statistics as reported by Quast

(b) (4)

Assembly Graph as Visualized by Bandage.

(b) (4)

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Appendix 004. Antimicrobial Susceptibility Report

Note for Appendix 004 regarding Table 1 on page 6 of the final report: MIC values in this dossier were compared to EFSA 2018 publication instead of 2012 as listed in the report. Below is a table comparing the EFSA 2012 MIC values vs ESFA 2018

Antimicrobial	MIC (µg/mL) of *Clostridium beijerinckii* ASCUSDY20 (Dairy-20)	2012 EFSA MIC (µg/mL) for other Gram +	2018 EFSA MIC (µg/mL) for other Gram +
Ampicillin	≤0.06	1	1
Chloramphenicol	16	2	4
Clindamycin	2	0.25	4
Erythromycin	1	0.5	1
Gentamicin	>32	4	4
Kanamycin	2	16	16
Streptomycin	8	0.5	8
Tetracycline	4	2	2
Vancomycin	1	2	4

FINAL REPORT

TITLE: **Characterization of Ascus Biosciences Dairy-20: Susceptibility Profile**

INVESTIGATOR'S STUDY NUMBER:

CONDUCT DATES:

Receipt of isolate: October 26, 2017
MIC Testing: October 25, 2017 – November 3, 2017

SPONSOR:

Ascus Biosciences
6450 Lusk Blvd
Suites E109/209
San Diego, CA 92121

INVESTIGATOR:

VERSION: FINAL

SIGNATURE: 1/15/18
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Principal Investigator   Date

TABLE OF CONTENTS

LIST OF TABLES AND APPENDICES

1. OBJECTIVES

To determine the susceptibility profile of the *Clostridium butyricum* (Dairy-20) production strain to multiple antimicrobials.

2. STANDARDS OF COMPLIANCE

This study was conducted in a GSP-like (Good Scientific Practice) manner in accordance with testing facility SOPs and to CLSI documents VET01 and M11 to the extent to which they were applicable as detailed in the protocol.

3. STUDY SITE

Susceptibility testing of the products was performed by   (b) (4)
(b) (4)

4. MATERIALS

The sponsor provided Dairy-20 production strain (i.e., *Clostridium butyricum*) was received on October 19, 2017. The culture was streaked to a trypticase soy agar with 5% sheep blood agar (BA) to verify that the organism was viable, pure and morphologically typical of the purported species.

5. SUSCEPTIBILITY PROFILE

5.1. Procedure

The procedures listed in this protocol were written to comply with CLSI document M11-A8 entitled Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard – Eighth Edition for anaerobes using the broth microdilution procedure.

MIC plates for were prepared by   (b) (4) with antimicrobials and doubling dilution concentrations as indicated in Figure 1 of the Protocol. The isolate was MIC tested according to   (b) (4)(Appendix A).

5.2. Media

Media for MIC testing was Supplemented Brucella Broth (SBB).

5.3.   Incubation and Interpretation of Susceptibility Tests

MIC plates were incubated and interpreted according to [redacted] (b) (4) for anaerobes (Appendix A).

5.4.   Quality Control

Bacteroides fragilis (ATCC 25285, [redacted] (b) (4)) and *Clostridium difficile* (ATCC 700057, [redacted] (b) (4)) was tested on each testing date. Available CLSI acceptable ranges for each of the quality control organism/antimicrobial combinations were referenced (Table 2).

With each testing date, one dilution series was inoculated with sterile broth to assess procedural and media sterility. Inoculum counts for bacteria were conducted according to [redacted] (b) (4).

6.   DISPOSITIONS

6.1.   The MIC plates were discarded after their expiration.

6.2.   The isolate, and all subcultures, were discarded after autoclaving. No retention culture was maintained.

7.   RESULTS

MIC results of the *Clostridium butyricum* (Dairy-20) isolate are presented in Table 1. The isolate would be considered wild-type or susceptible according to all three criteria (EFSA, EUCAST and CLSI, as available) to ampicillin, kanamycin and vancomycin. The isolate would be considered susceptible to ampicillin, clindamycin, and tetracycline according to CLSI and EUCAST breakpoints. According to EUCAST, the isolate would be resistant to chloramphenicol, although would be considered susceptible according to CLSI. The isolate would be considered non-wildtype, or non-susceptible, against chloramphenicol, clindamycin, erythromycin, gentamicin, streptomycin, and tetracycline. However, one must consider that the classifications set forth below are for general Gram positive organisms and may not be applicable to the *Clostridium* species tested in this study.

Table 1. Listing of MIC of Antimicrobials and EFSA Microbiological Cut-off Values and EUCAST and CLSI Breakpoints for Bacteria [1]

Antimicrobial	Tested Range (µg/mL)	MIC (µg/mL) of the *Clostridium butyricum* (Dairy-20)	EFSA Microbiological Cut-off Values (µg/mL) Other Gram +	EUCAST Resistant Breakpoints (µg/mL) Gram-positive anaerobes	CLSI Resistant Breakpoints (µg/mL) Anaerobes
Ampicillin	0.06-64	≤ 0.06	1	8	≥2
Chloramphenicol	2-32	16	2	8	≥32
Clindamycin	0.03-32	2	0.25	4	≥8
Erythromycin	0.25-8	1	0.5	NA	NA
Gentamicin	0.12-32	>32	4	NA	NA
Kanamycin	0.12-32	2	16	NA	NA
Streptomycin	0.12-32	8	0.5	NA	NA
Tetracycline	1-32	4	2	NA	≥16
Vancomycin	0.25-32	1	2	2	NA

[1] Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance; EFSA Journal 2012;10(6):2740. EUCAST breakpoints are for Gram-positive anaerobes, Clinical Breakpoint Tables V.7.1., CLSI M100S-26th Ed. Table 2J-1; NA=Not Available

The MIC results of the quality control organisms were within the expected values as indicated in Table 2. The inoculum counts were within specifications. Of note, the quality control acceptable range for ampicillin was incorrect in the protocol for the *Bacteroides fragilis* (ATCC 25285). The correct range is presented in Table 2.

Table 2. Quality Control Organism MIC Results

Antimicrobial	*Bacteroides fragilis* (Br-1) ATCC 25285 MIC	Acceptable Range[2]	*Clostridium difficile* (CL-16) ATCC 700057 MIC	Acceptable Range[2]
Ampicillin	(b) (4)	32-64	(b) (4)	1-4
Chloramphenicol		2-8		---
Clindamycin		0.5-2		2-8
Erythromycin		---		---
Gentamicin		---		---
Kanamycin		---		---
Streptomycin		---		---
Tetracycline		0.12-0.5		---
Vancomycin		---		0.5-4

[2] Obtained from CLSI document M100; --- = no range available.

APPENDIX A. Protocol and SOP Documents

(b) (4)

STUDY PROTOCOL

TITLE:	Characterization of Ascus Biosciences Dairy-20 and Dairy-21: Susceptibility Profile and Absence of Antimicrobial Activity
INVESTIGATOR'S STUDY NUMBER:	(b) (4)
SPONSOR:	Ascus Biosciences 6450 Lusk Blvd Suites E109/209 San Diego, CA 92121
INVESTIGATOR:	(b) (4)
VERSION:	FINAL: October 10, 2017

STUDY PROTOCOL No(s):   (b) (4)   Version FINAL 10/10/17
Characterization of Ascus Biosciences Dairy-20 and Dairy-21: Susceptibility Profile and Absence of
Antimicrobial Activity   Page 2 of 14

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Antimicrobial Activity   Page 3 of 14

SIGNATURES

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    Signature   Date

Investigator

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    (b) (4)

    _____   10/10/17
    Signature   Date

STUDY PROTOCOL No(s): [(b) (4)]   Version FINAL 10/10 17
Characterization of Ascus Biosciences Dairy-20 and Dairy-21: Susceptibility Profile and Absence of
Antimicrobial Activity   Page 3 of 14

SIGNATURES

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Signature   Date

STUDY PROTOCOL No(s): [(b) (4)]

Characterization of Ascus Biosciences Dairy-20 and Dairy-21: Susceptibility Profile and Absence of Antimicrobial Activity

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1. OBJECTIVES

   1.1. To determine the Susceptibility Profile of the *Clostridium butyricum* (Dairy-20) and *Pichia kudriavzevii* (Dairy-21) production strains to multiple antimicrobials.

   1.2. Determination of the antimicrobial properties of the *Clostridium butyricum* (Dairy-20) and *Pichia kudriavzevii* (Dairy-21) production strain supernatant.

2. STUDY TIMELINE

   Anticipated study dates are:
   Susceptibility Testing: November 2017
   Antimicrobial Properties: November 2017

3. STANDARDS OF COMPLIANCE

   This study will be conducted in a GSP-like (Good Scientific Practice) manner in accordance with testing facility SOPs and to CLSI documents VET01, M11 and M27 to the extent to which they are applicable as detailed in this protocol. European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints or epidemiological cutoff values (ECOFFs) may be referenced for determining non-wildtype MIC values. Procedures for the susceptibility were designed to follow those in European Food Safety Authority (EFSA) *Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance*[4] as applicable and as detailed in this protocol.

4. STUDY SITE

   Antimicrobial properties and susceptibility testing of the products will be performed by [(b) (4)]

5. MATERIALS AND METHODS

   5.1. Isolates

   The sponsor will provide the production strain and supernatant to test. The cultures will be streaked to an appropriate media (e.g., trypticase soy agar with 5% sheep blood agar (BA) for *Clostridium butyricum* and Sabouraud Dextrose Agar (SDA) for *Pichia kudriavzevii* to verify that the organisms are viable, pure and morphologically typical of the purported species.

---

[4] EFSA Journal 2012, 10(6): 2740

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5.2. Supernatants

The supernatants will be streaked onto BA or onto SDA as appropriate for the purported strain to verify their sterility. [(b) (4)]

6. SUSCEPTIBILITY PROFILE

6.1. Procedure

Each production strain will be tested. Additional strains may be tested upon direction of the sponsor. The MIC values of the *Clostridium butyricum* will be compared to the "other Gram +" cut-off values published by EFSA or CLSI/EUCAST breakpoints to determine if a non-wild type strain (defined as potentially harboring resistance mechanisms) or if non-susceptible [refer to Table 3]. The cut-off/non-susceptible values for *Pichia kudriavzevii* will be evaluated in a similar manner comparing to values from EUCAST ECOFF values or CLSI interpretive criteria (Table 4).

The procedures listed in this protocol were written to comply with CLSI document M11-A8 entitled Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard – Eighth Edition for anaerobes using the broth microdilution procedure. The yeast isolates was tested according to CLSI document M27-A3 entitled Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approve Standard – Third Edition with modification by the instruction sheet from the YeastOne panels (Thermo Scientific).

MIC plates for anaerobes will be prepared by [(b) (4)] with antimicrobials and doubling dilution concentrations as indicated in Figure 1. The yeast will be tested using Sensititre Yeast One susceptibility panels with the concentrations of antifungals listed in Figure 2. The isolates will be MIC tested according to [(b) (4)] for anaerobes or as indicated below for yeast.

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[(b) (4)]

6.2.   Media

Media for MIC testing will be Supplemented Brucella Broth (SBB) for anaerobes and
YeastOne broth (Thermo Fisher Scientific part number Y3462) for yeast.

6.3.   Incubation and Interpretation of Susceptibility Tests

MIC plates will be incubated and interpreted according to [(b) (4)] for anaerobes.
Refer to Section 6.1 for yeast.

6.4.   Quality Control

Bacteroides fragilis (ATCC 25285, [(b) (4)]) and *Clostridium difficile* (ATCC
700057, MRI code CL-16) will be tested on each testing date when testing anaerobes.
Candida parapsilosis (ATCC 220197, [(b) (4)]) and *Issatchenkia orientalis*
Kudrjanzev (ATCC 6258, [(b) (4)]) will be tested on each testing date when
testing yeast.

Below are listings of the available CLSI acceptable ranges for each of the quality
control organism/antimicrobial combinations.

With each testing date, one dilution series should be inoculated with sterile broth to
assess procedural and media sterility. Results of the sterility testing should be
recorded on the appropriate form. Conduct inoculum counts for bacteria according to
[(b) (4)]

Table 1.   Quality Control Organisms and Acceptable MIC ranges for Anaerobes[2]

	SBB	
Antimicrobial	*Bacteroides fragilis* (Br-1) ATCC 25285	*Clostridium difficile* (CL-16) ATCC 700057
Ampicillin	2-8	1-4
Chloramphenicol	2-8	---
Clindamycin	0.5-2	2-8
Erythromycin	---	---
Gentamicin	---	---
Kanamycin	---	---
Streptomycin	---	---
Tetracycline	0.12-0.5	---
Vancomycin	---	0.5-4

[2]Obtained from CLSI document M100

Table 2.   Quality Control Organisms and Acceptable MIC ranges for Yeast[3]

	MIC values for RPMIG (µg/mL)	
Antimicrobial	*Candida parapsilosis* ATCC 220197 (b) (4)	*Issatchenkia orientalis Kudrjanzev* (ATCC 6258 (b) (4)
Anidulafungin	0.25-2	0.03-0.12
Amphotericin B	0.25-2	0.5-2
Micafungin	0.5-2	0.12-0.5
Caspofungin	0.25-1	0.12-1
5-Flucytosine	0.06-0.25	4-16
Posaconazole	0.06-0.25	0.06-0.5
Voriconazole	0.016-0.12	0.06-0.5
Itraconazole	0.12-0.5	0.12-1
Fluconazole	0.5-4	8-64

[3]Obtained from CLSI document M27-S3 - 24Hr

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Table 3.   Listing of Antimicrobials and EFSA Microbiological Cut-off Values and EUCAST and CLSI Breakpoints for Bacteria [4]

Antimicrobial	Tested Range (µg/mL)	EFSA Microbiological Cut-off Values (µg/mL) Other Gram +	EUCAST Resistant Breakpoints (µg/mL) Gram-positive anaerobes	CLSI Resistant Breakpoints (µg/mL) Anaerobes
Ampicillin	0.06-64	1	8	≥2
Chloramphenicol	2-32	2	8	≥32
Clindamycin	0.03-32	0.25	4	≥8
Erythromycin	0.25-8	0.5	NA	NA
Gentamicin	0.12-32	4	NA	NA
Kanamycin	0.12-32	16	NA	NA
Streptomycin	0.12-32	0.5	NA	NA
Tetracycline	1-32	2	NA	≥16
Vancomycin	0.25-32	2	2	NA

[4] Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance; EFSA Journal 2012;10(6):2740. EUCAST breakpoints are for Gram-positive anaerobes, Clinical Breakpoint Tables V.7.1., CLSI M100S-26th Ed. Table 2J-1; NA=Not Available

Table 4.   Listing of Antimicrobials and EUCAST ECOFF and CLSI Interpretive Criteria Values for Yeast[4]

Antimicrobial	Tested Range (µg/mL)	EUCAST ECOFF Values (µg/mL)	CLSI non-susceptible or resistant Interpretive Criteria (µg/mL)
Anidulafungin	0.015-8	0.06	>2
Amphotericin B	0.12-8	1	Not Available
Micafungin	0.008-8	0.25	>2
Caspofungin	0.008-8	Not Available	>2
5-Flucytosine	0.06-64	Not Available	≥32
Posaconazole	0.008-8	Not Available	Not Available
Voriconazole	0.008-8	1	≥4
Itraconazole	0.015-16	1	≥1
Fluconazole	0.12-128	128	≥64

[4] EUCAST ECOFF values for *Candida krusei* (EUCAST) were accessed from https://mic.eucast.org/Eucast2/SearchController on 10/4/17 and CLSI M27-S3.

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7. ABSENCE OF ANTIMICROBIAL PRODUCTION[5]

The presence of antimicrobial activity in the growth medium from both production strains (Dairy-20 and Dairy-21) will be tested. A portion of the growth medium from a typical production batch of bacteria, or a scaled down version, will be centrifuged and the supernatant will be sterile filtered (0.45μm) by the sponsor. The supernatant will be kept refrigerated (2-8°C) and shipped to (b) (4) for use within 20 days. A minimum of 5 mL will be provided to (b) (4) Stability of the product will not be determined. Additional supernatants may be tested as directed by the sponsor.

7.1. Preparation of Culture Plates

The following six organisms will be tested against each supernatant:

Organism	ATCC number	(b) (4) code	Dilution tested
Staphylococcus aureus	6538	Sta 11	1:10
Escherichia coli	11229	EC 96	1:10
Bacillus cereus	2	BC 5	1:10
Bacillus circulans	4516	Bi 1	1:10
Streptococcus pyogenes	12344	Str 59	1:20
Serratia marcescens	14041	SM 4	1:10

(b) (4)

---

[5] FAO (1999) Determination of Antibacterial Activity of enzyme preparations from the Combined Compendium of Food Additive Specifications, Vol. 4 (FAO/JECFA), pg 122.

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7.2. Disk Preparation

(b) (4)

7.3. Incubation

(b) (4)

7.4. Interpretation

(b) (4)

7.5. Quality Control

(b) (4)

8. RAW DATA, RECORDS, AND REPORTS

8.1. Data
All raw data will be recorded, handled, and stored according to facility SOPs, this
protocol, and applicable regulatory requirements. All original data collected and

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records generated in connection with the study will be archived at the study site. The following records will be maintained:

➢ Quality control records generated concurrent with all media and materials preparation, and lab testing,

➢ Protocols, protocol amendments, correspondence, reports and other documentation, including drafts of the final report

➢ Raw data and logs

➢ Documents related to any occurrence or situation that develops during the course of the trial that may affect the test results

All records will be maintained appropriately in labs and files as the project is ongoing, and thereafter in archives storage at (b) (4)

8.2. REPORTING OF RESULTS

A separate report will be issued for the production strain for each of the tests performed. Hence, a total of 4 reports will be issued according to the following Table:

Production Strain	Report required (X) for the indicated test description for the Protocol section	
	1: Susceptibility Profile	2: Antimicrobial Activity
Clostridium butyricum	X	X
Pichia kudriavzevii	X	X

If additional production strains are tested, reports will be issued in a similar manner, depending upon the tests required.

9. DISPOSITIONS

9.1. All surplus quantities of the provided supernatants will be discarded after autoclaving following report issue. No reserve samples will be maintained.

9.2. MIC plates will be discarded after their expiration.

9.3. Isolates will be discarded after autoclaving. No retention cultures will be maintained.

10. CHANGES TO PROTOCOL

Any change or revision to the approved protocol will be documented by written amendment that will be maintained with the protocol. As a minimum, the amendment will indicate the changes or revisions made, indicate the effective date, identify the protocol sections affected, explain the reasons for change and describe the impact on the study. The amendment will be

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signed and dated by those who signed the protocol. Signatures will be obtained before
implementation of the change if possible. If such is not possible, the investigator will
attempt to obtain verbal prior authorization from the sponsor and follow with written
documentation at the earliest opportunity. Protocol deviations are defined as unintended or
unforeseeable necessary changes to the protocol. Protocol deviation reports list any action
that is not/was not in accordance with the protocol. They must contain a detailed description
of the deviation, its reason, and a description of its effect on the study.

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Figure 1.   MIC Plate Diagram for (b) (4) Prepared Plate (1 isolate per plate)

Abbreviation	Antimicrobial
AMP	Ampicillin
CLI	Clindamycin
CHL	Chloramphenicol
ERY	Erythromycin
GEN	Gentamicin
POS CTRL	Inoculated, non-antimicrobial, positive control growth well

Abbreviation	Antimicrobial
KAN	Kanamycin
STR	Streptomycin
TET	Tetracycline
VAN	Vancomycin
NEG CTRL	Uninoculated, non-antimicrobial, negative control well

(numerals indicate the concentration in µg/mL contained within the well)

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Figure 2. MIC Plate Diagram for Yeast One Sensititre Plates (1 isolate per plate)

	1	2	3	4	5	6	7	8	9	10	11	12
A	POS CTRL	AND 0.015	AND 0.03	AND 0.06	AND 0.12	AND 0.25	AND 0.5	AND 1	AND 2	AND 4	AND 8	AB 0.12
B	MF 0.008	MF 0.015	MF 0.03	MF 0.06	MF 0.12	MF 0.25	MF 0.5	MF 1	MF 2	MF 4	MF 8	AB 0.25
C	CAS 0.008	CAS 0.015	CAS 0.03	CAS 0.06	CAS 0.12	CAS 0.25	CAS 0.5	CAS 1	CAS 2	CAS 4	CAS 8	AB 0.5
D	FC 0.06	FC 0.12	FC 0.25	FC 0.5	FC 1	FC 2	FC 4	FC 8	FC 16	FC 32	FC 64	AB 1
E	PZ 0.008	PZ 0.015	PZ 0.03	PZ 0.06	PZ 0.12	PZ 0.25	PZ 0.5	PZ 1	PZ 2	PZ 4	PZ 8	AB 2
F	VOR 0.008	VOR 0.015	VOR 0.03	VOR 0.06	VOR 0.12	VOR 0.25	VOR 0.5	VOR 1	VOR 2	VOR 4	VOR 8	AB 4
G	IZ 0.015	IZ 0.03	IZ 0.06	IZ 0.12	IZ 0.25	IZ 0.5	IZ 1	IZ 2	IZ 4	IZ 8	IZ 16	AB 8
H	FZ 0.12	FZ 0.25	FZ 0.5	FZ 1	FZ 2	FZ 4	FZ 8	FZ 16	FZ 32	FZ 64	FZ 128	FZ 256

Abbreviation	Antimicrobial
AND	Anidulafungin
AB	Amphotericin B
MF	Micafungin
CAS	Caspofungin
FC	5-Flucytosine
POS CTRL	Inoculated, non-antimicrobial, positive control growth well

Abbreviation	Antimicrobial
PZ	Posaconazole
VOR	Voriconazole
IZ	Itraconazole
FZ	Fluconazole

(numerals indicate the concentration in μg/mL contained within the well)

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SOP [(b) (4)]
Revision #4
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SUSCEPTIBILITY TESTING OF ANAEROBES

Section 1. General Considerations

The procedures described herein are designed to follow those described in CLSI document M11.

Disk diffusion testing is not addressed in the CLSI document or in this SOP, and should be performed only for a specific purpose. No QC ranges are available for disk diffusion tests.

[(b) (4)]

Section 2. Inoculum preparation

[(b) (4)]

SOP [(b) (4)]
Revision #4
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SUSCEPTIBILITY TESTING OF ANAEROBES

Section 3. Agar Dilution

1. Media

[(b) (4)]

2. Inoculation of plates

[(b) (4)]

3. Reading plates

[(b) (4)]

SOP [(b) (4)]
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SUSCEPTIBILITY TESTING OF ANAEROBES

Interpret the MIC endpoint as the lowest concentration where a marked reduction occurs in the appearance of growth as compared to the anaerobic control plates. Refer to CLSI document M11 for example interpretations as needed.

Section 4. Broth microdilution – MRI prepared plates

1. Preparation of plates

(b) (4)

2. Inoculation of plates

(b) (4)

3. Interpretation of MIC

The growth endpoint will be recorded as the concentration where the most significant reduction of growth is observed. Examples of growth patterns and endpoint determinations are provided in CLSI document M11.

SOP [(b) (4)]
Revision #4
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SUSCEPTIBILITY TESTING OF ANAEROBES

Section 5. Broth microdilution – Sensititre

1. Inoculation of plates

[(b) (4)]

2. Interpretation of MIC

[(b) (4)]

Section 6. Quality Control Tests

1. Organisms

The protocol or SSP for a specific project should be referenced for selection of QC organisms to test. Consult the laboratory director for the specific organisms to test if a listing of the isolates is unavailable. The QC organism may include one or more of the following: *Bacteroides fragilis* (ATCC 25285, [(b) (4)], *Bacteroides thetaiotaomicron* (ATCC 29741, [(b) (4)], and *Clostridium difficile* (ATCC 700057, [(b) (4)].

2. Acceptable ranges

The acceptable ranges will be obtained from CLSI document M11 or as provided by the sponsor. As directed by protocol, SSP or laboratory director, internal QC ranges can be developed according to QC-49.

SOP ▨ (b) (4)
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SUSCEPTIBILITY TESTING OF ANAEROBES

3. Sterility checks

   Sterility checks will be included for both agar dilution and broth microdilution using sterile broth inoculated into a dilution series.

4. Inoculum counts

   Inoculum counts will be performed as described in SOP ▨ (b) (4)

Reference:

CLSI Publication No. M11-A8, "Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard – Eighth Edition," Vol. 32, No. 5.

Prepared by: ▨ (b) (4), (b) (6)

Date: 11/24/14
Reference
Verification by: ▨ (b) (4), (b) (6)

Date: 12/8/14

Reviewed by ▨ (b) (4), (b) (6)

Date: 12/9/14
Approved by: ▨ (b) (4), (b) (6)

Date: 12/9/14
Effective Date: 12/23/14

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SOP ░░░░ (b) (4)
Revision #9
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ACCURACY VERIFICATION OF STANDARDIZED CULTURES FOR SUSCEPTIBILITY TESTING

Section 1. General

Weekly, for each procedure described herein (when susceptibility tests are being conducted), after standardized and diluted test isolates have been inoculated according to the appropriate susceptibility procedure, the inoculum density will be tested to assure that the procedures for standardizing and diluting inoculum remain under control. Randomly select 5% of the susceptibility tests (up to five). One of the accuracy verification tests should be conducted on a QC organism in order to assist in determining the effect of an out-of-range colony count. If a study is performed in which no QC organisms are included alongside test isolates, the accuracy verification tests will be performed on test isolates only.

Calculations of the expected colony counts are based on the count of a 0.5 McFarland standard averaging 1.5×10^8 CFU/mL, and allow for errors associated with diluting and loss of viability occurring during diluting and plating. In general, the ranges for acceptable colony counts were developed to allow for ±1 LOG of target concentration.

A nutritive broth medium may be substituted for saline in any of the dilutions described in this SOP. For the more fastidious organisms, it is advisable to use a nutritive broth in lieu of saline (e.g., reduced Brucella broth [BB] or Brain Heart Infusion broth (BHI) should be used as the diluent for anaerobic bacteria).

Due to the difficulty of maintaining anaerobiosis while handling anaerobic organisms during the dilution process, and the slow growth patterns of anaerobic organisms, the resulting counts may be inaccurate. These counts will be recorded but will not be used to accept or reject the results of the susceptibility testing. If anaerobic organism counts are consistently low, the dilution procedure may be changed to account for loss of viability by handling the organisms during the inoculation procedure.

Tests should be conducted within 10 minutes of performing the susceptibility procedure.

Section 2. Agar Dilution

    A. General:

(b) (4)

SOP [redacted] (b) (4)
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ACCURACY VERIFICATION OF STANDARDIZED CULTURES FOR SUSCEPTIBILITY TESTING

[redacted] (b) (4)

[redacted] (b) (4)

B. Anaerobic Bacteria:

[redacted] (b) (4)

SOP (b) (4)
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ACCURACY VERIFICATION OF STANDARDIZED CULTURES
FOR SUSCEPTIBILITY TESTING

For *Clostridium difficile:*

(b) (4)

Section 3. Disk Diffusion and/or E Test

(b) (4)

Section 4. Microdilution (MRI prepared MIC plates and/or Sensititre)

A. Bacteria

(b) (4)

SOP (b) (4)
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ACCURACY VERIFICATION OF STANDARDIZED CULTURES
FOR SUSCEPTIBILITY TESTING

B. Mycoplasma

(b) (4)

C. Anaerobic Bacteria

(b) (4)

SOP [(b) (4)]
Revision #9
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ACCURACY VERIFICATION OF STANDARDIZED CULTURES FOR SUSCEPTIBILITY TESTING

Section 5. Interpretation of Results

If QC organism and test isolates fall within the acceptable range, data will be recorded.

If any counts fall out of the ranges described in Sections 2, 3 or 4, the Laboratory Director should be notified to determine if the low or high count is severe enough to have an effect on test isolates.

If results consistently show counts to be unacceptable, the following should be checked to determine cause and the corrective action to take:

- McFarland Standard quality: Prepare fresh standards if needed.
- Are test isolates being properly compared to McFarland standard? Re-train personnel if needed.
- Proper culture dilution: Re-train personnel if needed, and/or adjust dilution procedure.
- Pipettor set to the correct volume; adjust if necessary.
- Is the proper dilution and plating procedure being followed as described in this SOP? Re-train personnel if needed.

If counts for a particular organism are consistently out of range, the dilution procedure for preparation of inoculum of that particular organism should be adjusted after determining actual counts of a 0.5 McFarland standard adjusted suspension of that organism.

Prepared by: _____ [(b) (4)]

Date: 5/31/16 _____

Reviewed by: _____ [(b) (4)]

Date: 5/31/16 _____

Approved by: _____ [(b) (4)]

Date: 5/31/16 _____
Effective Date: 6/14/16 _____

FINAL REPORT

TITLE: **Characterization of Ascus Biosciences Dairy-20: Antibacterial Properties**

INVESTIGATOR'S STUDY NUMBER: (b) (4)

CONDUCT DATES: Receipt of supernatant: October 25, 2017
Testing of supernatant: November 8, 2017 – November 11, 2017

SPONSOR: Ascus Biosciences
6450 Lusk Blvd
Suites E109/209
San Diego, CA 92121

INVESTIGATOR: (b) (4)

VERSION: FINAL

SIGNATURE: (b) (4)   1/15/18
Date

Principal Investigator

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LIST OF TABLES AND APPENDICES

OBJECTIVES

To determine the antimicrobial properties of the *Clostridium butyricum* (Dairy-20) production strain supernatant.

STANDARDS OF COMPLIANCE

This study was conducted in a GSP-like (Good Scientific Practice) manner in accordance with testing facility SOPs as detailed in the protocol.

STUDY SITE

Antimicrobial property testing of the product was performed by [redacted]   (b) (4)

MATERIALS

The sponsor provided Dairy-20 (Lot AS101817fA) supernatant was prepared by centrifugation at 25,000RPM for 15 minutes followed by sterile filtration with a 0.2um membrane. The sample was received on October 25, 2017.

ANTIMICROBIAL PROPERTIES

A portion of the growth medium from a typical production batch of bacteria, or a scaled down version, was centrifuged and the supernatant sterile filtered by the sponsor. The supernatant was kept refrigerated (2-8°C) and shipped to MRI and used 16 days after preparation.

1.1. Preparation of Culture Plates

The following six organisms were tested against the supernatant:

Organism	ATCC number	(b) (4) code	Dilution tested
Staphylococcus aureus	6538	Sta 11	1:10
Escherichia coli	11229	EC 96	1:10
Bacillus cereus	2	BC 5	1:10
Bacillus circulans	4516	Bi 1	1:10
Streptococcus pyogenes	12344	Str 59	1:20
Serratia marcescens	14041	SM 4	1:10

(b) (4)

1.2. Disk Preparation

(b) (4)

1.3. Incubation

(b) (4)

1.4. Interpretation

(b) (4)

1.5. Quality Control

(b) (4)

DISPOSITIONS

The supernatant was discarded after autoclaving and issue of the final report. No retention sample was maintained.

RESULTS

No zones of inhibition were observed for Dairy-20 supernatant lot, or the sterile distilled water control. A zone of inhibition was observed for the enrofloxacin positive control for each organism as indicated in the table below:

Table 1. Zone Diameters from Dairy-20 Supernatant and Controls

Organism	ATCC number	(b) (4) code	Zone Diameter for the indicated solution (mm)		
			Dairy-20 Lot: AS101817fA	Sterile Distilled water	Enrofloxacin
Staphylococcus aureus	6538	Sta 11			(b) (4)
Escherichia coli	11229	EC 96			
Bacillus cereus	2	BC 5			
Bacillus circulans	4516	Bi 1			
Streptococcus pyogenes	12344	Str 59			
Serratia marcescens	14041	SM 4			

Following incubation, pictures were taken of each organism seeded into the agar onto which a saturated disk of supernatant and controls were placed according to the protocol. These pictures are included in Appendix B. No zones of inhibition are observed in these pictures.

CONCLUSION

The Dairy-20 supernatant (Lot AS101817fA) exhibited no antibacterial activity against the 6 strains representative of Gram positive and Gram negative bacteria.

APPENDIX A. Protocol

(b) (4)

STUDY PROTOCOL

TITLE:	**Characterization of Ascus Biosciences Dairy-20 and Dairy-21: Susceptibility Profile and Absence of Antimicrobial Activity**
INVESTIGATOR'S STUDY NUMBER:	(b) (4)
SPONSOR:	Ascus Biosciences 6450 Lusk Blvd Suites E109/209 San Diego, CA 92121
INVESTIGATOR:	(b) (4)
VERSION:	FINAL: October 10, 2017

STUDY PROTOCOL No(s):   (b) (4)   Version FINAL 10/10/17
Characterization of Ascus Biosciences Dairy-20 and Dairy-21: Susceptibility Profile and Absence of
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Characterization of Ascus Biosciences Dairy-20 and Dairy-21: Susceptibility Profile and Absence of
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SIGNATURES

Sponsor
Representative   Adam Taylor
                          Ascus Biosciences
                          6450 Lusk Blvd
                          Suites E109/209
                          San Diego, CA 92121
                          Email: adam@ascusbiosciences.com
                          Tel. 707-601-2553

_____   _____
Signature   Date

Investigator   (b) (4)

                                                        (b) (4)
                                                        1 0/10/17
_____   _____
Signature   Date

STUDY PROTOCOL No(s): [(b) (4)]   Version FINAL 10/10/17
Characterization of Ascus Biosciences Dairy-20 and Dairy-21: Susceptibility Profile and Absence of
Antimicrobial Activity   Page 3 of 14

SIGNATURES

Sponsor
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                          _____   10/10/17
                          Signature   Date

Investigator   (b) (4)

                          _____   _____
                          Signature   Date

STUDY PROTOCOL No(s):   [b) (4)]   Version FINAL 10/10/17
Characterization of Ascus Biosciences Dairy-20 and Dairy-21: Susceptibility Profile and Absence of
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1. OBJECTIVES

    1.1. To determine the Susceptibility Profile of the *Clostridium butyricum* (Dairy-20) and
    Pichia kudriavzevii (Dairy-21) production strains to multiple antimicrobials.

    1.2. Determination of the antimicrobial properties of the *Clostridium butyricum*
    (Dairy-20) and *Pichia kudriavzevii* (Dairy-21) production strain supernatant.

2. STUDY TIMELINE

    Anticipated study dates are:
    Susceptibility Testing: November 2017
    Antimicrobial Properties: November 2017

3. STANDARDS OF COMPLIANCE

    This study will be conducted in a GSP-like (Good Scientific Practice) manner in accordance
    with testing facility SOPs and to CLSI documents VET01, M11 and M27 to the extent to
    which they are applicable as detailed in this protocol. European Committee on Antimicrobial
    Susceptibility Testing (EUCAST) breakpoints or epidemiological cutoff values (ECOFFs)
    may be referenced for determining non-wildtype MIC values. Procedures for the
    susceptibility were designed to follow those in European Food Safety Authority (EFSA)
    *Guidance on the assessment of bacterial susceptibility to antimicrobials of human and
    veterinary importance*[4] as applicable and as detailed in this protocol.

4. STUDY SITE

    Antimicrobial properties and susceptibility testing of the products will be performed by
    [b) (4)]

5. MATERIALS AND METHODS

    5.1. Isolates

    The sponsor will provide the production strain and supernatant to test. The cultures
    will be streaked to an appropriate media (e.g., trypticase soy agar with 5% sheep
    blood agar (BA) for *Clostridium butyricum* and Sabouraud Dextrose Agar (SDA) for
    Pichia kudriavzevii to verify that the organisms are viable, pure and morphologically
    typical of the purported species.

---

[4] EFSA Journal 2012, 10(6): 2740

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5.2.   Supernatants

The supernatants will be streaked onto BA or onto SDA as appropriate for the
purported strain to verify their sterility.   (b) (4)

6. SUSCEPTIBILITY PROFILE

6.1.   Procedure

Each production strain will be tested. Additional strains may be tested upon direction
of the sponsor. The MIC values of the *Clostridium butyricum* will be compared to the
"other Gram +" cut-off values published by EFSA or CLSI/EUCAST breakpoints to
determine if a non-wild type strain (defined as potentially harboring resistance
mechanisms) or if non-susceptible [refer to Table 3]. The cut-off/non-susceptible
values for *Pichia kudriavzevii* will be evaluated in a similar manner comparing to
values from EUCAST ECOFF values or CLSI interpretive criteria (Table 4).

The procedures listed in this protocol were written to comply with CLSI document
M11-A8 entitled Methods for Antimicrobial Susceptibility Testing of Anaerobic
Bacteria; Approved Standard – Eighth Edition for anaerobes using the broth
microdilution procedure. The yeast isolates was tested according to CLSI document
M27-A3 entitled Reference Method for Broth Dilution Antifungal Susceptibility
Testing of Yeasts; Approve Standard – Third Edition with modification by the
instruction sheet from the YeastOne panels (Thermo Scientific).

MIC plates for anaerobes will be prepared by   (b) (4)   with
antimicrobials and doubling dilution concentrations as indicated in Figure 1. The
yeast will be tested using Sensititre Yeast One susceptibility panels with the
concentrations of antifungals listed in Figure 2. The isolates will be MIC tested
according to   (b) (4)   for anaerobes or as indicated below for yeast.

(b) (4)

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6.2.   Media

Media for MIC testing will be Supplemented Brucella Broth (SBB) for anaerobes and
YeastOne broth (Thermo Fisher Scientific part number Y3462) for yeast.

6.3.   Incubation and Interpretation of Susceptibility Tests

MIC plates will be incubated and interpreted according to [(b) (4)] for anaerobes.
[(b) (4)]

6.4.   Quality Control

Bacteroides fragilis (ATCC 25285, [(b) (4)] and *Clostridium difficile* (ATCC
700057, [(b) (4)] will be tested on each testing date when testing anaerobes.
Candida parapsilosis (ATCC 220197, [(b) (4)] and *Issatchenkia orientalis
Kudrjanzev* (ATCC 6258, MRI code IO-1) will be tested on each testing date when
testing yeast.

Below are listings of the available CLSI acceptable ranges for each of the quality
control organism/antimicrobial combinations.

With each testing date, one dilution series should be inoculated with sterile broth to
assess procedural and media sterility. Results of the sterility testing should be
recorded on the appropriate form. Conduct inoculum counts for bacteria according to
[(b) (4)]

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Table 1.   Quality Control Organisms and Acceptable MIC ranges for Anaerobes[2]

	SBB	
Antimicrobial	*Bacteroides fragilis* (Br-1) ATCC 25285	*Clostridium difficile* (CL-16) ATCC 700057
Ampicillin	2-8	1-4
Chloramphenicol	2-8	---
Clindamycin	0.5-2	2-8
Erythromycin	---	---
Gentamicin	---	---
Kanamycin	---	---
Streptomycin	---	---
Tetracycline	0.12-0.5	---
Vancomycin	---	0.5-4

[2]Obtained from CLSI document M100

Table 2.   Quality Control Organisms and Acceptable MIC ranges for Yeast[3]

	MIC values for RPMIG (µg/mL)	
Antimicrobial	*Candida parapsilosis* ATCC 220197, MRI code CR-1	*Issatchenkia orientalis Kudrjanzev* (ATCC 6258, MRI code IO-1)
Anidulafungin	0.25-2	0.03-0.12
Amphotericin B	0.25-2	0.5-2
Micafungin	0.5-2	0.12-0.5
Caspofungin	0.25-1	0.12-1
5-Flucytosine	0.06-0.25	4-16
Posaconazole	0.06-0.25	0.06-0.5
Voriconazole	0.016-0.12	0.06-0.5
Itraconazole	0.12-0.5	0.12-1
Fluconazole	0.5-4	8-64

[3]Obtained from CLSI document M27-S3 · 24Hr

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Table 3.   Listing of Antimicrobials and EFSA Microbiological Cut-off Values
            and EUCAST and CLSI Breakpoints for Bacteria [4]

Antimicrobial	Tested Range (µg/mL)	EFSA Microbiological Cut-off Values (µg/mL) Other Gram +	EUCAST Resistant Breakpoints (µg/mL) Gram-positive anaerobes	CLSI Resistant Breakpoints (µg/mL) Anaerobes
Ampicillin	0.06-64	1	8	≥ 2
Chloramphenicol	2-32	2	8	≥ 32
Clindamycin	0.03-32	0.25	4	≥ 8
Erythromycin	0.25-8	0.5	NA	NA
Gentamicin	0.12-32	4	NA	NA
Kanamycin	0.12-32	16	NA	NA
Streptomycin	0.12-32	0.5	NA	NA
Tetracycline	1-32	2	NA	≥ 16
Vancomycin	0.25-32	2	2	NA

[4] Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary
importance; EFSA Journal 2012;10(6):2740. EUCAST breakpoints are for Gram-positive
anaerobes, Clinical Breakpoint Tables V.7.1., CLSI M100S-26th Ed. Table 2J-1; NA=Not
Available

Table 4.   Listing of Antimicrobials and EUCAST ECOFF and CLSI Interpretive
            Criteria Values for Yeast[4]

Antimicrobial	Tested Range (µg/mL)	EUCAST ECOFF Values (µg/mL)	CLSI non-susceptible or resistant Interpretive Criteria (µg/mL)
Anidulafungin	0.015-8	0.06	>2
Amphotericin B	0.12-8	1	Not Available
Micafungin	0.008-8	0.25	>2
Caspofungin	0.008-8	Not Available	>2
5-Flucytosine	0.06-64	Not Available	≥ 32
Posaconazole	0.008-8	Not Available	Not Available
Voriconazole	0.008-8	1	≥ 4
Itraconazole	0.015-16	1	≥ 1
Fluconazole	0.12-128	128	≥ 64

[4] EUCAST ECOFF values for *Candida krusei* (EUCAST) were accessed from
https://mic.eucast.org/Eucast2/SearchController on 10/4/17 and CLSI M27-S3.

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7. ABSENCE OF ANTIMICROBIAL PRODUCTION[5]

The presence of antimicrobial activity in the growth medium from both production strains
(Dairy-20 and Dairy-21) will be tested. (b) (4)

7.1. Preparation of Culture Plates

The following six organisms will be tested against each supernatant:

Organism	ATCC number	(b) (4) code	Dilution tested
Staphylococcus aureus	6538	Sta 11	1:10
Escherichia coli	11229	EC 96	1:10
Bacillus cereus	2	BC 5	1:10
Bacillus circulans	4516	Bi 1	1:10
Streptococcus pyogenes	12344	Str 59	1:20
Serratia marcescens	14041	SM 4	1:10

(b) (4)

---

[5] FAO (1999) Determination of Antibacterial Activity of enzyme preparations from the Combined Compendium of
Food Additive Specifications, Vol. 4 (FAO/JECFA), pg 122.

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    7.2.   Disk Preparation

(b) (4)

    7.3.   Incubation

(b) (4)

    7.4.   Interpretation

(b) (4)

    7.5.   Quality Control

(b) (4)

8. RAW DATA, RECORDS, AND REPORTS

    8.1.   Data
        All raw data will be recorded, handled, and stored according to facility SOPs, this
        protocol, and applicable regulatory requirements. All original data collected and

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Characterization of Ascus Biosciences Dairy-20 and Dairy-21: Susceptibility Profile and Absence of
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records generated in connection with the study will be archived at the study site. The
following records will be maintained:

> Quality control records generated concurrent with all media and materials
  preparation, and lab testing,
> Protocols, protocol amendments, correspondence, reports and other doc-
  umentation, including drafts of the final report
> Raw data and logs
> Documents related to any occurrence or situation that develops during the course
  of the trial that may affect the test results

All records will be maintained appropriately in labs and files as the project is
ongoing, and thereafter in archives storage at   (b) (4)

8.2.   REPORTING OF RESULTS

A separate report will be issued for the production strain for each of the tests
performed. Hence, a total of 4 reports will be issued according to the following
Table:

Production Strain	Report required (X) for the indicated test description for the Protocol section	
	1: Susceptibility Profile	2: Antimicrobial Activity
Clostridium butyricum	X	X
Pichia kudriavzevii	X	X

If additional production strains are tested, reports will be issued in a similar manner,
depending upon the tests required.

9. DISPOSITIONS

9.1.   All surplus quantities of the provided supernatants will be discarded after autoclaving
following report issue. No reserve samples will be maintained.

9.2.   MIC plates will be discarded after their expiration.

9.3.   Isolates will be discarded after autoclaving. No retention cultures will be maintained.

10. CHANGES TO PROTOCOL

Any change or revision to the approved protocol will be documented by written amendment
that will be maintained with the protocol. As a minimum, the amendment will indicate the
changes or revisions made, indicate the effective date, identify the protocol sections affected,
explain the reasons for change and describe the impact on the study. The amendment will be

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Characterization of Ascus Biosciences Dairy-20 and Dairy-21: Susceptibility Profile and Absence of
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signed and dated by those who signed the protocol. Signatures will be obtained before implementation of the change if possible. If such is not possible, the investigator will attempt to obtain verbal prior authorization from the sponsor and follow with written documentation at the earliest opportunity. Protocol deviations are defined as unintended or unforeseeable necessary changes to the protocol. Protocol deviation reports list any action that is not/was not in accordance with the protocol. They must contain a detailed description of the deviation, its reason, and a description of its effect on the study.

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Figure 1.   MIC Plate Diagram for (b) (4) Prepared Plate (1 isolate per plate)

	1	2	3	4	5	6	7	8	9	10	11	12
A	CLI 0.06	CLI 0.12	CLI 0.25	CLI 0.5	CLI 1	CLI 2	CLI 4	CLI 8	CLI 16	CLI 32	AMP 8	
B	CHL 0.06	CHL 0.12	CHL 0.25	CHL 0.5	CHL 1	CHL 2	CHL 4	CHL 8	CHL 16	CHL 32	AMP 4	
C	ERY 0.06	ERY 0.12	ERY 0.25	ERY 0.5	ERY 1	ERY 2	ERY 4	ERY 8	ERY 16	ERY 32	AMP 2	
D	GEN 0.06	GEN 0.12	GEN 0.25	GEN 0.5	GEN 1	GEN 2	GEN 4	GEN 8	GEN 16	GEN 32	AMP 1	
E	KAN 0.06	KAN 0.12	KAN 0.25	KAN 0.5	KAN 1	KAN 2	KAN 4	KAN 8	KAN 16	KAN 32	AMP 0.5	AMP 32
F	STR 0.06	STR 0.12	STR 0.25	STR 0.5	STR 1	STR 2	STR 4	STR 8	STR 16	STR 32	AMP 0.25	AMP 16
G	TET 0.06	TET 0.12	TET 0.25	TET 0.5	TET 1	TET 2	TET 4	TET 8	TET 16	TET 32	AMP 0.12	POS CTRL
H	VAN 0.06	VAN 0.12	VAN 0.25	VAN 0.5	VAN 1	VAN 2	VAN 4	VAN 8	VAN 16	VAN 32	AMP 0.06	NEG CTRL

Abbreviation	Antimicrobial		Abbreviation	Antimicrobial
AMP	Ampicillin		KAN	Kanamycin
CLI	Clindamycin		STR	Streptomycin
CHL	Chloramphenicol		TET	Tetracycline
ERY	Erythromycin		VAN	Vancomycin
GEN	Gentamicin			
POS CTRL	Inoculated, non-antimicrobial, positive control growth well		NEG CTRL	Uninoculated, non-antimicrobial, negative control well
	(numerals indicate the concentration in µg/mL contained within the well)			

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Figure 2. MIC Plate Diagram for Yeast One Sensititre Plates (1 isolate per plate)
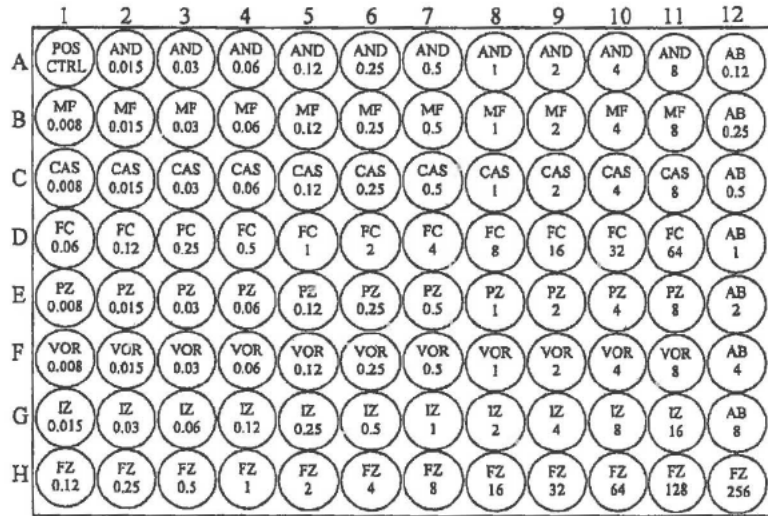
Abbreviation	Antimicrobial		Abbreviation	Antimicrobial
AND	Anidulafungin		PZ	Posaconazole
AB	Amphotericin B		VOR	Voriconazole
MF	Micafungin		IZ	Itraconazole
CAS	Caspofungin		FZ	Fluconazole
FC	5-Flucytosine			
POS CTRL	Inoculated, non-antimicrobial, positive control growth well			
(numerals indicate the concentration in µg/mL contained within the well)				

APPENDIX B: Photos
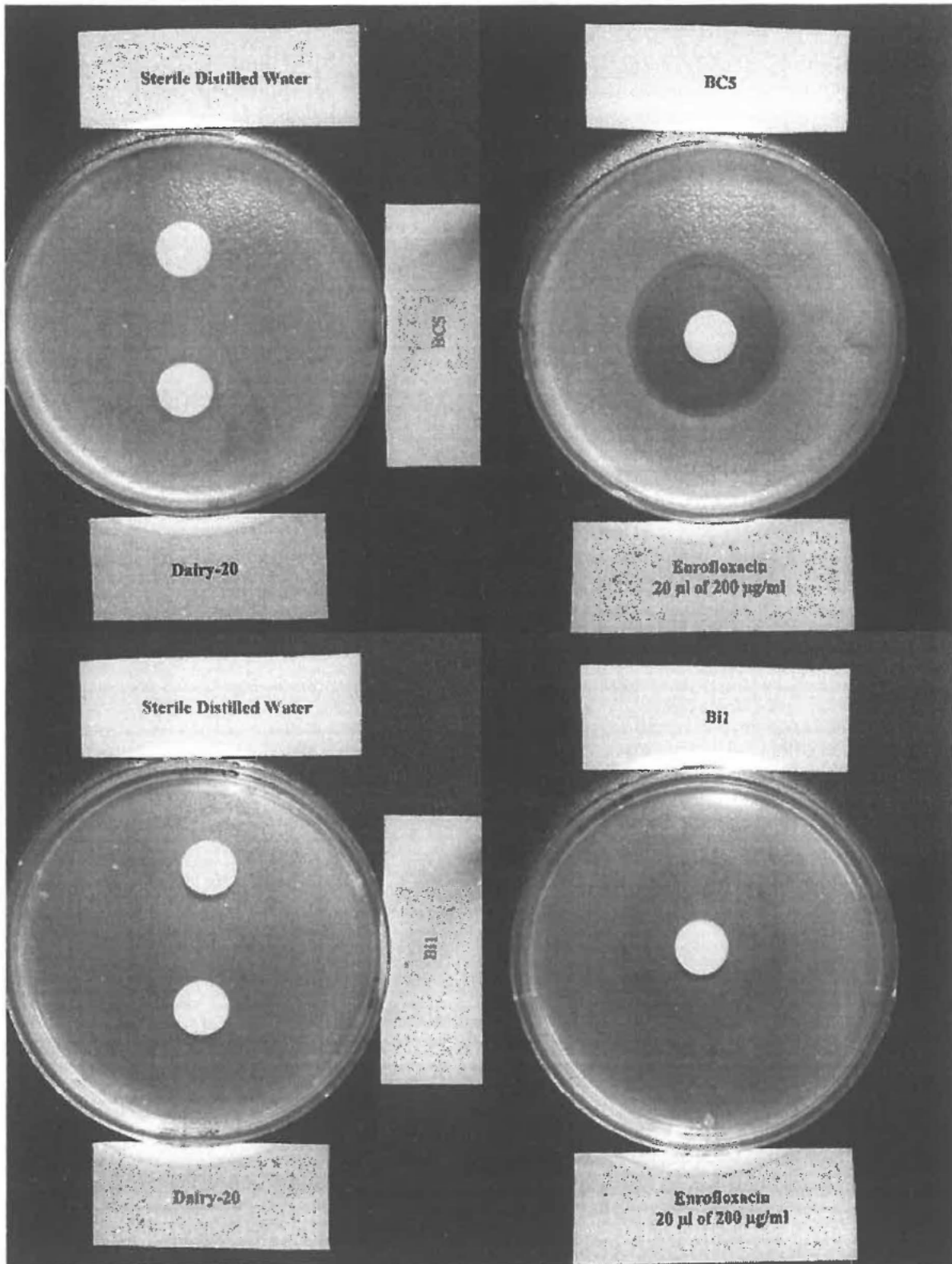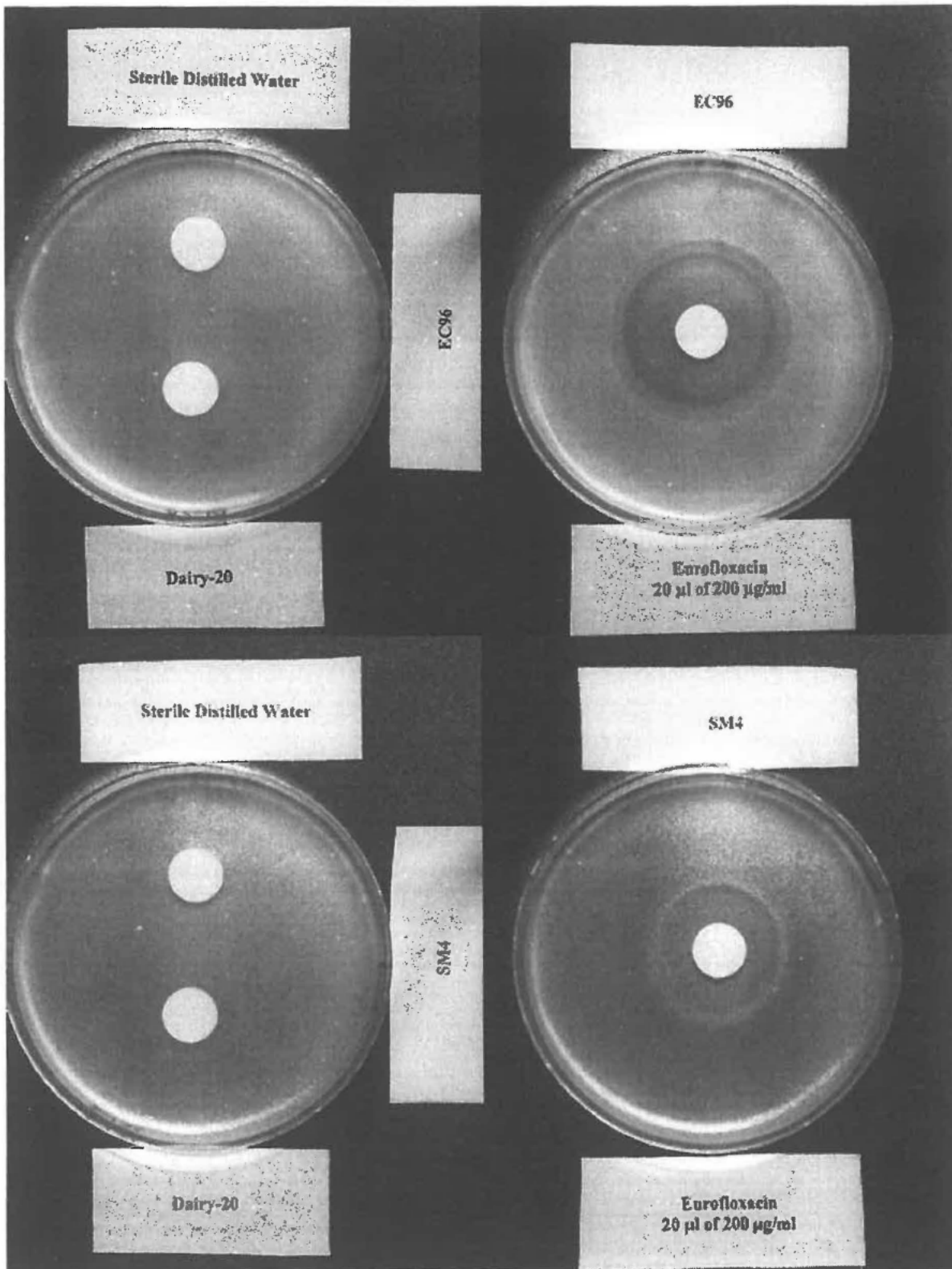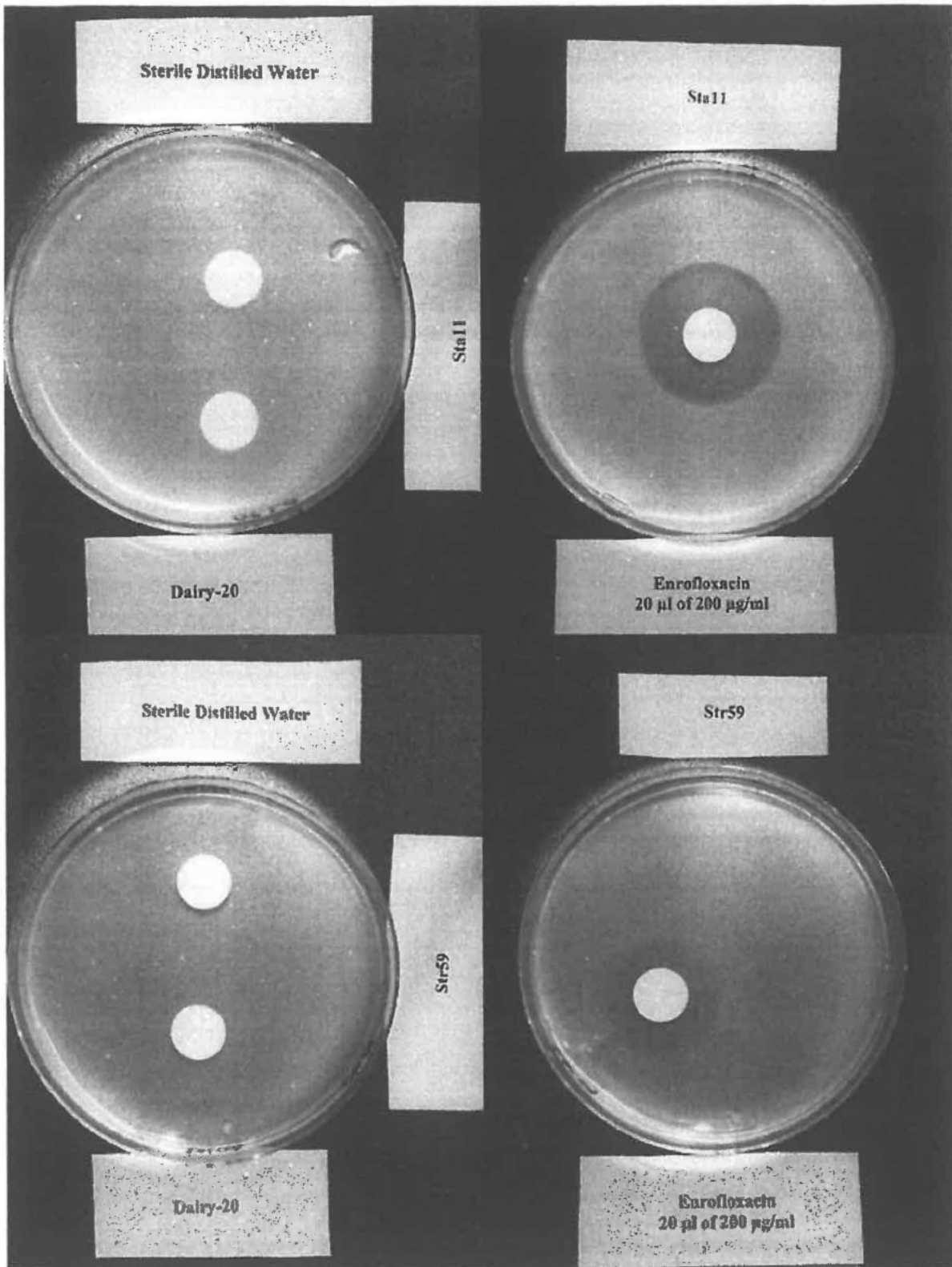
ULINE S-18139

FOOD BAGS

1-800-295-5510
uline.com
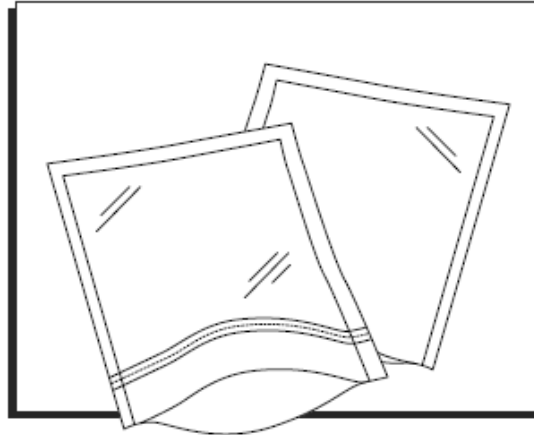
PRODUCT SPECIFICATIONS

Uline Food Bags provide protection against moisture and corrosion for packaging of powders and food-related products. Uline Food Bags are FDA approved.

PHYSICAL FILM PROPERTIES	UNITS
Moisture Vapor Transmission Rate (ASTM F1249)	0.0003 gr./100 in^2 24 hrs.
O$_2$ Transmission Rate (73.4°F, 0% RH)(ASTM D 3985-05)	0.001/cc/m^2/24 hrs.
Bursting Strength (TAPPI T403)	69 psi.
Puncture (FED. 101)	> 18 psi.
Total Thickness	4.4 mil

Uline makes no warranty, expressed or implied, as to the suitability of these materials for any specific use. The values shown above were developed from random samples taken from production material. We believe them to be typical for the product. Actual values may vary somewhat from those depicted here. Customers should determine product suitability based upon their own internal criteria.

ULINE S-18139

BOLSAS PARA ALIMENTOS

800-295-5510
uline.mx
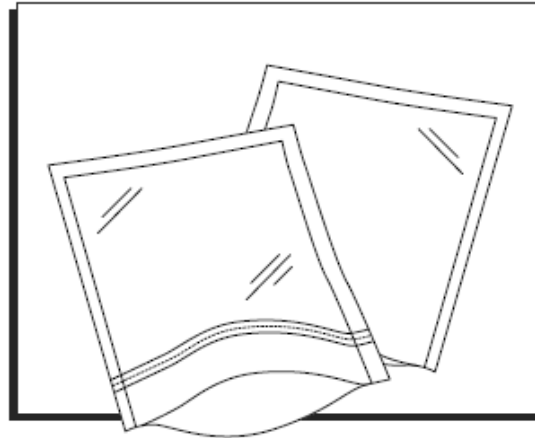
ESPECIFICACIONES DEL PRODUCTO

Las bolsas para alimentos de Uline brindan protección ante la humedad y la corrosión para el empaque de productos en polvo y relacionados con la alimentación. Las bolsas para alimentos de Uline han sido aprobadas por la FDA.

PROPIEDADES FÍSICAS DE LA PELÍCULA	UNIDADES
Tasa de Transmisión de Vapor de Agua (ASTM F1249)	0.0003 gr./100 pul.² 24 h
Tasa de transmisión de O₂ [23°C (73.4°F), 0% RH](ASTM D 3985-05)	0.001/cc/m²/24 hrs.
Resistencia al Reventamiento (TAPPI T403)	69 psi.
Perforaciones (FED. 101)	> 18 psi.
Grosor Total	4.4 mil

Uline no garantiza de forma alguna, ya sea implícita o explícita, la idoneidad de estos materiales para cualquier uso específico. Los valores arriba mostrados han sido desarrollados a partir de muestras aleatorias tomadas del material de producción. Creemos que son típicos del producto. Los valores reales podrían diferir con respecto a los mostrados aquí. Los clientes deberán determinar la idoneidad del producto de acuerdo con su propio criterio interno.

ULINE S-18139

SACS POUR ALIMENTS

1-800-295-5510
uline.ca
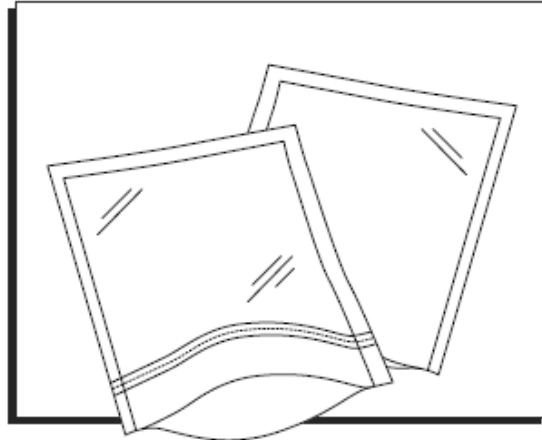
SPÉCIFICATIONS DU PRODUIT

Les sacs pour aliments Uline permettent d'emballer des produits alimentaires ou en poudre pour les protéger de l'humidité et de la corrosion. Les sacs pour aliments Uline sont approuvés par la FDA.

PROPRIÉTÉS PHYSIQUES DE LA PELLICULE	UNITÉS
Taux de transmission de vapeur humide (ASTM F1249)	0,0003 gr./100 po² 24 h
Taux de transmission d'O₂ [23 °C (73.4 °F), 0 % RH](ASTM D 3985-05)	0,001/cc/m²/24 h
Résistance à l'éclatement (TAPPI T403)	69 lb/po²
Perforation (FED. 101)	> 18 lb/po²
Épaisseur totale	4,4 mil

Uline n'offre aucune garantie, explicite ou implicite, quant à la convenance de ces matériaux pour tout usage précis. Les valeurs indiquées ci-dessus ont été élaborées à partir d'échantillons prélevés au hasard dans la production. Nous croyons qu'elles sont représentatives du produit. Les valeurs réelles peuvent varier quelque peu de celles qui sont énoncées ici. La convenance du produit à un usage précis reste à être déterminée par le client lui-même.

BAM: Aerobic Plate Count

January 2001

Bacteriological Analytical Manual
Chapter 3
Aerobic Plate Count

Authors: Larry Maturin (ret.) and James T. Peeler (ret)

For additional information, contact Guodong Zhang (mailto:guodong.zhang@fda.hhs.gov).

Chapter Contents

- Conventional Plate Count Method

- Spiral Plate Method

- References

The aerobic plate count (APC) is intended to indicate the level of microorganism in a product. Detailed procedures for determining the APC of foods have been developed by the Association of Official Analytical Chemists (AOAC) (3) and the American Public Health Association (APHA) (1). The conventional plate count method for examining frozen, chilled, precooked, or prepared foods, outlined below, conforms to AOAC *Official Methods of Analysis*, sec. 966.23, with one procedural change (966.23C). The suitable colony counting range (10) is 25-250. The automated spiral plate count method for the examination of foods and cosmetics (5), outlined below, conforms to AOAC *Official Methods of Analysis*, sec. 977.27. For procedural details of the standard plate count, see ref. 2.Guidelines for calculating and reporting plate counts have been changed to conform with the anticipated changes in the 16th edition of *Standard Methods for the Examination of Dairy Products* (2) and the *International Dairy Federation* (IDF) procedures (6).

Conventional Plate Count Method

A. **Equipment and materials**

1. Work area, level table with ample surface in room that is clean, well-lighted (100 foot-candles at working surface) and well-ventilated, and reasonably free of dust and drafts. The microbial density of air in working area, measured in fallout pour plates taken during plating, should not exceed 15 colonies/plate during 15 min exposure.

2. Storage space, free of dust and insects and adequate for protection of equipment and supplies

3. Petri dishes, glass or plastic (at least 15 × 90 mm)

4. Pipets with pipet aids (no mouth pipetting) or pipettors, 1, 5, and 10 ml, graduated in 0.1 ml units

5. Dilution bottles, 6 oz (160 ml), borosilicate-resistant glass, with rubber stoppers or plastic screw caps

6. Pipet and petri dish containers, adequate for protection

7. Circulating water bath, for tempering agar, thermostatically controlled to 45 ± 1°C

8. Incubator, 35 ± 1°C; milk, 32 ± 1°C

9. Colony counter, dark-field, Quebec, or equivalent, with suitable light source and grid plate

10. Tally register

11. Dilution blanks, 90 ± 1 ml Butterfield's phosphate-buffered dilution water (R11 (/food/laboratory-methods/bam-r11-butterfields-phosphate-buffered-dilution-water)); milk, 99 ± 2 ml

12. Plate count agar (standard methods) (M124 (/food/laboratory-methods/bam-media-m124-plate-count-agar-standard-methods))

13. Refrigerator, to cool and maintain samples at 0-5°C; milk, 0-4.4°C

14. Freezer, to maintain frozen samples from -15 to -20°C

15. Thermometers (mercury) appropriate range; accuracy checked with a thermometer certified by the National Institute of Standards and Technology (NIST)

B. **Procedure for analysis of frozen, chilled, precooked, or prepared foods**

Using separate sterile pipets, prepare decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and others as appropriate, of food homogenate (**see** Chapter 1 (/food/laboratory-methods/bam-food-samplingpreparation-sample-homogenate) for sample preparation) by transferring 10 ml of previous dilution to 90 ml of diluent. Avoid sampling foam. Shake all dilutions 25 times in 30 cm (1 ft) arc within 7 s. Pipet 1 ml of each dilution into separate, duplicate, appropriately marked petri dishes. Reshake dilution bottle 25 times in 30 cm arc within 7 s if it stands more than 3 min before it is pipetted into petri dish. Add 12-15 ml plate count agar (cooled to 45 ± 1°C) to each plate within 15 min of original dilution. For milk samples, pour an agar control, pour a dilution water control and pipet water for a pipet control. Add agar to the latter two for each series of samples. Add agar immediately to petri dishes when sample diluent contains hygroscopic materials, e.g., flour and starch. Pour agar and dilution water control plates for each series of samples. Immediately mix sample dilutions and agar medium

thoroughly and uniformly by alternate rotation and back-and-forth motion of plates on flat level surface. Let agar solidify. Invert solidified petri dishes, and incubate promptly for 48 ± 2 h at 35°C. Do not stack plates when pouring agar or when agar is solidifying.

C. **Guidelines for calculating and reporting APCs in uncommon cases**

Official Methods of Analysis (3) does not provide guidelines for counting and reporting plate counts, whereas *Standard Methods for the Examination of Dairy Products*, 16th ed. (2) presents detailed guidelines; for uniformity, therefore, use APHA guidelines as modified (6,8). Report all aerobic plate counts (2) computed from duplicate plates. For milk samples, report all aerobic plate (2) counts computed from duplicate plates containing less than 25 colonies as less than 25 estimated count. Report all aerobic plate counts (2) computed from duplicate plates containing more than 250 colonies as estimated counts. Counts outside the normal 25-250 range may give erroneous indications of the actual bacterial composition of the sample. Dilution factors may exaggerate low counts (less than 25), and crowded plates (greater than 250) may be difficult to count or may inhibit the growth of some bacteria, resulting in a low count. Report counts less than 25 or more than 250 colonies as estimated aerobic plate counts (EAPC). Use the following guide:

1. Normal plates (25-250). Select spreader-free plate(s). Count all colony forming units (CFU), including those of pinpoint size, on selected plate(s). Record dilution(s) used and total number of colonies counted.

2. Plates with more than 250 colonies. When number of CFU per plate exceeds 250, for all dilutions, record the counts as too numerous to count (TNTC) for all but the plate closest to 250, and count CFU in those portions of plate that are representative of colony distribution. See ref. 2 for detailed guidelines. Mark calculated APC with EAPC to denote that it was estimated from counts outside 25-250 per plate range (*see* D-3).

3. Spreaders. Spreading colonies are usually of 3 distinct types: 1) a chain of colonies, not too distinctly separated, that appears to be caused by disintegration of a bacterial clump; 2) one that develops in film of water between agar and bottom of dish; and 3) one that forms in film of water at edge or on surface of agar. If plates prepared from sample have excessive spreader growth so that (a) area covered by spreaders, including total area of repressed growth, exceeds 50% of plate area, or (b) area of repressed growth exceeds 25% of plate area, report plates as spreaders. When it is necessary to count plates containing spreaders not eliminated by (a) or (b) above, count each of the 3 distinct spreader types as one source. For the first type, if only one chain exists, count it as a single colony. If one or more chains appear to originate from separate sources, count each source as one colony. Do not count each individual growth in such chains as a separate colony. Types 2 and 3 usually result in distinct colonies and are counted as such. Combine the spreader count and the colony count to compute the APC.

4. Plates with no CFU. When plates from all dilutions have no colonies, report APC as less than 1 times the corresponding lowest dilution used. Mark calculated APC with asterisk to denote that it was estimated from counts outside the 25-250 per plate range. When plate(s) from a sample are known to be contaminated or otherwise unsatisfactory, record the result(s) as laboratory accident (LA).

D. **Computing and recording counts (see refs 6, 8)**

To avoid creating a fictitious impression of precision and accuracy when computing APC, report only the first two significant digits. Round off to two significant figures only at the time of conversion to SPC. For milk samples, when plates for all dilutions have no colonies, report APC as less than 25 colonies estimated count. Round by raising the second digit to the next highest number when the third digit is 6, 7, 8, or 9 and use zeros for each successive digit toward the right from the second digit. Round down when the third digit is 1, 2, 3, or 4. When the third digit is 5, round up when the second digit is odd and round down when the second digit is even.

Examples

Calculated Count	APC
12,700	13,000
12,400	12,000
15,500	16,000
14,500	14,000

1. Plates with 25-250 CFU.

$$N = \frac{\sum C}{[(1 \times n_1) + (0.1 \times n_2) \times (d)]}$$

a. Calculate the APC as follows:

$$\frac{(31+31)\ \text{colonies}}{0.0015\,\text{ml}} = 4.1 \times 10^4$$

= 537/0.022

= 24,409

≈ 24,000

b. When counts of duplicate plates fall within and without the 25-250 colony range, use only those counts that fall within this range.

2. All plates with fewer than 25 CFU. When plates from both dilutions yield fewer than 25 CFU each, record actual plate count but record the count as less than 25 × 1/d when d is the dilution factor for the dilution from which the first counts were obtained.

Example

Colonies		
1:100	**1:1000**	**EAPC/ml (g)**
18	2	<>
0	0	<>

3. **All plates with more than 250 CFU**. When plates from both 2 dilutions yield more than 250 CFU each (but fewer than 100/cm²), estimate the aerobic counts from the plates (EAPC) nearest 250 and multiply by the dilution.

   Example

Colonies		
1:100	**1:1000**	**EAPC/ml (g)**
TNTC	640	640,000

   TNTC, too numerous to count.
   EAPC, estimated aerobic plate count.

4. All plates with spreaders and/or laboratory accident. Report respectively as Spreader (SPR), or Laboratory Accident (LA).

5. All plates with more than an average of 100 CFU per sq cm. Estimate the APC as greater than 100 times the highest dilution plated, times the area of the plate. The examples below have an average count of 110 per sq cm.

   Example

Colonies/Dilution		
1:100	**1:1000**	**EAPC/ml (g)**
TNTC	7,150[a]	>6,500,000 EAPC[b]
TNTC	6,490	>5,900,000 EAPC

[a] Based on plate area of 65 cm²
[b] EAPC, estimated APC
[c] Based on plate area of 59 cm²

Spiral Plate Method

The spiral plate count (SPLC) method for microorganisms in milk, foods, and cosmetics is an official method of the APHA (2) and the AOAC (3). In this method, a mechanical plater inoculates a rotating agar plate with liquid sample. The sample volume dispensed decreases as the dispensing stylus moves from the center to the edge of the rotating plate. The microbial concentration is determined by counting the colonies on a part of the petri dish where they are easily countable and dividing this count by the appropriate volume. One inoculation determines microbial densities between 500 and 500,000 microorganisms/ml. Additional dilutions may be made for suspected high microbial concentrations.

A. **Equipment and materials**

1. Spiral plater (Spiral Systems Instruments, Inc., 7830 Old Georgetown Road, Bethesda, MD 20814)

2. Spiral colony counter (Spiral Systems) with special grid for relating deposited sample volumes to specific portions of petri dishes

3. Vacuum trap for disposal of liquids (2-4 liter vacuum bottle to act as vacuum reservoir and vacuum source of 50-60 cm Hg)

4. Disposable micro beakers, 5 ml

5. Petri dishes, plastic or glass, 150 × 15 mm or 100 × 15 mm

6. Plate count agar (standard methods) (M124 (/food/laboratory-methods/bam-media-m124-plate-count-agar-standard-methods))

7. Calculator (optional), inexpensive electronic hand calculator is recommended

8. Polyethylene bags for storing prepared plates

9. Commercial sodium hypochlorite solution, about 5% NaOCl (bleach)

10. Sterile dilution water

11. Syringe, with Luer tip for obstructions in stylus; capacity not critical

12. Work area, storage space, refrigerator, thermometers, tally, incubator, as described for Conventional Plate Count Method, above.

13. Sodium hypochlorite solution (5.25%). Available commercially.

B. **Preparation of agar plates.**

Automatic dispenser with sterile delivery system is recommended to prepare agar plates. Agar volume dispensed into plates is reproducible and contamination rate is low compared to hand-pouring of agar in open laboratory. When possible, use laminar air flow hood along with automated dispenser. Pour same quantity of agar into all plates so that same height of agar will be presented to spiral plater stylus tip to maintain contact angle. Agar plates should be level during cooling.

The following method is suggested for prepouring agar plates: Use automatic dispenser or pour constant amount (about 15 ml/100 mm plate; 50 ml/150 mm plate) of sterile agar at 60-70°C into each petri dish. Let agar solidify on level surface with poured

plates stacked no higher than 10 dishes. Place solidified agar plates in polyethylene bags, close with ties or heat-sealer, and store inverted at 0-4.4°C. Bring prepoured plates to room temperature before inoculation.

C. **Preparation of samples.**

As described in Chapter 1, select that part of sample with smallest amount of connective tissues or fat globules.

D. **Description of spiral plater.**

Spiral plater inoculates surface of prepared agar plate to permit enumeration of microorganisms in solutions containing between 500 and 500,000 microorganisms per ml. Operator with minimum training can inoculate 50 plates per h. Within range stated, dilution bottles or pipets and other auxiliary equipment are not required. Required bench space is minimal, and time to check instrument alignment is less than 2 min. Plater deposits decreasing amount of sample in Archimedean spiral on surface of prepoured agar plate. Volume of sample on any portion of plate is known. After incubation, colonies appear along line of spiral. If colonies on a portion of plate are sufficiently spaced from each other, count them on special grid which associates a calibrated volume with each area. Estimate number of microorganisms in sample by dividing number of colonies in a defined area by volume contained in same area. Studies have shown the method to be proficient not only with milk (4) but also with other foods (7,10).

E. **Plating procedure**

Check stylus tip angle daily and adjust if necessary. (Use vacuum to hold microscope cover slip against face of stylus tip; if cover slip plane is parallel at about l mm from surface of platform, tip is properly oriented). Liquids are moved through system by vacuum. Clean stylus tip by rinsing for 1 s with sodium hypochlorite solution followed by sterile dilution water for 1 s before sample introduction. This rinse procedure between processing of each sample minimizes cross-contamination. After rinsing, draw sample into tip of Teflon tubing by vacuum applied to 2-way valve. When tubing and syringe are filled with sample, close valve attached to syringe. Place agar plate on platform, place stylus tip on agar surface, and start motor. During inoculation, label petri plate lid. After agar has been inoculated, stylus lifts from agar surface and spiral plater automatically stops. Remove inoculated plate from platform and cover it. Move stylus back to starting position. Vacuum-rinse system with hypochlorite and water, and then introduce new sample. Invert plates and promptly place them in incubator for 48 ± 3 h at 35 ± 1°C.

F. **Sterility controls**

Check sterility of spiral plater for each series of samples by plating sterile dilution water. CAUTION: Prepoured plates should not be contaminated by a surface colony or be below room temperature (water can well-up from agar). They should not be excessively dry, as indicated by large wrinkles or glazed appearance. They should not

have water droplets on surface of agar or differences greater than 2 mm in agar depth, and they should not be stored at 0-4.4°C for longer than l month. Reduced flow rate through tubing indicates obstructions or material in system. To clear obstructions, remove valve from syringe, insert hand-held syringe with Luer fitting containing water, and apply pressure. Use alcohol rinse to remove residual material adhering to walls of system. Dissolve accumulated residue with chromic acid. Rinse well after cleaning.

G. **Counting grid**

1. **Description.** Use same counting grid for both 100 and 150 mm petri dishes. A mask is supplied for use with 100 mm dishes. Counting grid is divided into 8 equal wedges; each wedge is divided by 4 arcs labeled l, 2, 3, and 4 from outside grid edge. Other lines within these arcs are added for ease of counting. A segment is the area between 2 arc lines within a wedge. Number of areas counted (e.g., 3) means number of segments counted within a wedge. Spiral plater deposits sample on agar plate in the same way each time. The grid relates colonies on spiral plate to the volume in which they were contained. When colonies are counted with grid, sample volume becomes greater as counting starts at outside edge of plate and proceeds toward center of plate.

2. **Calibration.** The volume of sample represented by various parts of the counting grid is shown in operator's manual that accompanies spiral plater. Grid area constants have been checked by the manufacturer and are accurate. To verify these values, prepare 11 bacterial concentrations in range of 10^6-10^3 cells/ml by making 1:1 dilutions of bacterial suspension (use a nonspreader). Plate all Incubate both sets of plates for 48 ± 3 h at 35 ± 1°C. Calculate concentrations for each dilution. Count spiral plates over grid surface, using counting rule of 20 (described in H, below), and record number of colonies counted and grid area over which they were counted. Each spiral colony count for a particular grid area, divided by aerobic count/ml for corresponding spirally plated bacterial concentrations, indicates volume deposited on that particular grid area. Use the following formula:

$$\text{Volume (ml) for grid area} = \frac{\text{Spiral Colonies counted in area}}{\text{Baterial count/ml (APC)}}$$

$$\text{Volume (ml)} = \frac{31 + 30 \text{ colonies}}{4.1 \times 10^4 \text{ bateria/ml}} = 0.0015 \text{ ml}$$

To check total volume dispensed by spiral plater, weigh amount dispensed from stylus tip. Collect in tared 5 ml plastic beaker and weigh on analytical balance (± 0.2 mg).

Fig. 1 10 cm plate

Figure 1. 10 cm plate, area (3b)

$$\frac{(31+31) \text{ colonies}}{0.0015 \text{ ml}} = 4.1 \times 10^4$$

H. Examination and reporting of spiral plate counts.

Counting rule of 20. After incubation, center spiral plate over grid by adjusting holding arms on viewer. Choose any wedge and begin counting colonies from outer edge of first segment toward center until 20 colonies have been counted. Complete by counting remaining colonies in segment where 20th colony occurs. In this counting procedure, numbers such as 3b, 4c (Fig. l) refer to area segments from outer edge of wedge to designated arc line. Any count irregularities in sample composition are controlled by counting the same segments in the opposite wedge and recording results. Example of spirally inoculated plate (Fig. l) demonstrates method for determining microbial count. Two segments of each wedge were counted on opposite sides of plate with 31 and 30 colonies, respectively. The sample volume contained in the darkened segments is 0.0015 ml. To estimate number of microorganisms, divide count by volume contained in all segments counted. See example under Fig. l.

If 20 CFU are not within the 4 segments of the wedge, count CFU on entire plate. If the number of colonies exceeds 75 in second, third, or fourth segment, which also contains the 20th colony, the estimated number of microorganisms will generally be low because of coincidence error associated with crowding of colonies. In this case, count each circumferentially adjacent segment in all 8 wedges, counting at least 50 colonies, e.g., if the first 2 segments of a wedge contain 19 colonies and the third segment contains the 20th and 76th (or more), count colonies in all circumferentially adjacent first and second segments in all 8 wedges. Calculate contained volume in counted segments of wedges and divide into number of colonies.

When fewer than 20 colonies are counted on the total plate, report results as "less than 500 estimated SPLC per ml." If colony count exceeds 75 in first segment of wedge, report results as "greater than 500,000 estimated SPLC per ml." Do not count spiral plates with irregular distribution of colonies caused by dispensing errors. Report results of such plates as laboratory accident (LA). If spreader covers entire plate, discard plate. If spreader covers half of plate area, count only those colonies that are well distributed in spreader-free areas.

Compute SPLC unless restricted by detection of inhibitory substances in sample, excessive spreader growth, or laboratory accidents. Round off counts as described in I-D, above. Report counts as SPLC or estimated SPLC per ml.

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AOAC Official Method 2015.01
Heavy Metals in Food
Inductively Coupled Plasma–Mass Spectrometry
First Action 2015

Note: The following is not intended to be used as a comprehensive training manual. Analytical procedures are written based on the assumption that they will be performed by technicians who are formally trained in at least the basic principles of chemical analysis and in the use of the subject technology.

{Applicable for the determination of heavy metals [arsenic (As), CAS No. 7440-38-2; cadmium (Cd), CAS No. 7440-43-9; lead (Pb), CAS No. 7439-92-1; and mercury (Hg), CAS No. 7439-97-6] at trace levels in food and beverage samples, including solid chocolate, fruit juice, fish, infant formula, and rice, using microwave digestion and inductively coupled plasma–mass spectrometry (ICP-MS).}

Caution: Nitric acid and hydrochloric acid are corrosive. When working with these acids, wear adequate protective gear, including eye protection, gloves with the appropriate resistance, and a laboratory coat. Use an adequate fume hood for all acids.

Hydrogen peroxide is a strong oxidizer and can react violently with organic material to give off oxygen gas and heat. Adequate protective gear should be worn.

Many of the chemicals have toxicities that are not well established and must be handled with care. For all known chemicals used, consult the Material Safety Data Sheet (MSDS) in advance.

The inductively coupled plasma–mass spectrometer emits UV light when the plasma is on. UV resistant goggles should be worn if working near the plasma.

The instrument generates high levels of radio frequency (RF) energy and is very hot when the plasma is on. In the case of an instrument failure, be aware of these potential dangers.

Safely store interference reduction technology (IRT) gases, such as oxygen, in a closed, ventilated cabinet. Use adequate caution with pressurized gases. Prior training or experience is necessary to change any gas cylinders. Oxygen gas can cause many materials to ignite easily.

Following microwave digestion, samples are hot to the touch. Allow the samples to cool to room temperature before opening the digestion vessels to avoid unexpected depressurization and potential release of toxic fumes.

A. Principle

Food samples are thoroughly homogenized and then prepared by microwave digestion and the addition of dilute solutions of gold (Au) and lutetium (Lu). The Au is used to stabilize the Hg in the preparation, and the Lu is used to assess the potential loss of analyte during the microwave digestion process.

A prepared, diluted, aqueous sample digestate is pumped through a nebulizer, where the liquid forms an aerosol as it enters a spray chamber. The aerosol separates into a fine aerosol mist and larger aerosol droplets. The larger droplets exit the spray chamber while the fine mist is transported into the ICP torch.

Inside the ICP torch, the aerosol mist is transported into a high-temperature plasma, where it becomes atomized and ionized as it passes through an RF load coil. The ion stream is then focused by a single ion lens through a cylinder with a carefully controlled electrical field. For instruments equipped with dynamic reaction cell (DRC) or collision cell IRT, the focused ion stream is directed into the reaction/collision cell where, when operating with a pressurized cell, the ion beam will undergo chemical modifications and/or collisions to reduce elemental interferences. When not operating with a pressurized cell, the ion stream will remain focused as it passes through the cell with no chemical modification taking place.

The ion stream is then transported to the quadrupole mass filter, where only ions having a desired mass-to-charge ratio (m/z) are passed through at any moment in time. The ions exiting the mass filter are detected by a solid-state detector and the signal is processed by the data handling system.

B. Equipment

Perform routine preventative maintenance for the equipment used in this procedure.

An ultra-clean laboratory environment is critical for the successful production of quality data at ultra-low levels. All sample preparation must take place in a clean hood (Class 100). Metallic materials should be kept to a minimum in the laboratory and coated with an acrylic polymer gel where possible. Adhesive floor mats should be used at entrances to the laboratory and changed regularly to prevent the introduction of dust and dirt from the outside environment. Wear clean-room gloves and change whenever contact is made with anything non-ultra-clean. The laboratory floor should be wiped regularly to remove any particles without stirring up dust. *Note:* "Ultra-clean" (tested to be low in the analytes of interest) reagents, laboratory supplies, facilities, and sample handling techniques are required to minimize contamination in order to achieve the trace-level detection limits described herein.

(**a**) *Instrumentation*.—ICP-MS instrument, equipped with IRT with a free-running 40 MHz RF generator; and controllers for nebulizer, plasma, auxiliary, and reaction/collision flow control. The quadrupole mass spectrometer has a mass range of 5 to 270 atomic mass units (amu). The turbo molecular vacuum system achieves 10^{-6} torr or better. Recommended ICP-MS components include an RF coil, platinum skimmer and sampler cones, Peltier-cooled quartz cyclonic spray chamber, quartz or sapphire injector, micronebulizer, variable speed peristaltic pump, and various types of tubing (for gases, waste, and peristaltic pump). *Note*: The procedure is written specifically for use with a PerkinElmer ELAN DRC II ICP-MS (www.perkinelmer.com). Equivalent procedures may be performed on any type of ICP-MS instrument with equivalent IRT if the analyst is fully trained in the interpretation of spectral and matrix interferences and procedures for their correction, including the optimization of IRT. For example, collision cell IRT can be used for arsenic determination using helium gas.

(**b**) *Gases*.—High-purity grade liquid argon (>99.996%). Additional gases are required for IRT (such as ultra-x grade, 99.9999% minimum purity oxygen, used for determination of As in DRC mode with some PerkinElmer ICP-MS instruments).

(**c**) *Analytical balance*.—Standard laboratory balance suitable for sample preparation and capable of measuring to 0.1 mg.

(**d**) *Clean-room gloves*.—Tested and certified to be low in the metals of interest.

(**e**) *Microwave digestion system.*—Laboratory microwave digestion system with temperature control and an adequate supply of chemically inert digestion vessels. The microwave should be appropriately vented and corrosion resistant.

(*1*) The microwave digestion system must sense the temperature to within ±2.5°C and automatically adjust the microwave field output power within 2 s of sensing. Temperature sensors should be accurate to ±2°C (including the final reaction temperature of 190°C). Temperature feedback control provides the primary control performance mechanism for the method.

(*2*) The use of microwave equipment with temperature feedback control is required to control the unfamiliar reactions of unique or untested food or beverage samples. These tests may require additional vessel requirements, such as increased pressure capabilities.

(**f**) *Autosampler cups.*—15 and 50 mL; vials are precleaned by soaking in 2–5% (v/v) HNO_3 overnight, rinsed three times with reagent water/deionized water (DIW), and dried in a laminar flow clean hood. For the 50 mL vials, as these are used to prepare standards and bring sample preparations to final volume, the bias and precision of the vials must be assessed and documented prior to use. The recommended procedure for this is as follows:

(*1*) For every case of vials from the same lot, remove 10 vials.

(*2*) Tare each vial on an analytical balance, and then add reagent water up to the 20 mL mark. Repeat procedure by adding reagent water up to the 50 mL mark.

(*3*) Measure and record the mass of reagent water added, and then calculate the mean and RSD of the 10 replicates at each volume.

(*4*) To evaluate bias, the mean of the measurements must be with ±3% of the nominal volume. To evaluate precision, the RSD of the measurements must be ≤3% using the stated value (20 or 50 mL) in place of the mean.

(**g**) *Spatulas.*—To weigh out samples; should be acid-cleaned plastic (ideally Teflon) and cleaned by soaking in 2% (v/v) HNO_3 prior to use.

C. Reagents and Standards

Reagents may contain elemental impurities that could negatively affect data quality. High-purity reagents should always be used. Each reagent lot should be tested and certified to be low in the elements of interest before use.

(**a**) *DIW.*—ASTM Type I; demonstrated to be free from the metals of interest and potentially interfering substances.

(**b**) *Nitric acid (HNO_3).*—Concentrated; tested and certified to be low in the metals of interest.

(**c**) *Hydrogen peroxide (H_2O_2).*—Optima grade or equivalent, 30–32% assay.

(**d**) *Stock standard solutions.*—Obtained from a reputable and professional commercial source.

(*1*) *Single-element standards.*—Obtained for each determined metal, as well as for any metals used as internal standards and interference checks.

(*2*) *Second source standard.*—Independent from the single-element standard; obtained for each determined metal.

(*3*) *Multi-element stock standard solution.*—Elements must be compatible and stable in solutions together. Stability is determined by the vendor; concentrations are then verified before use of the standard.

(**e**) *Internal standard solution.*—For analysis of As, Cd, Pb, and Hg in food matrices, an internal standard solution of 40 µg/L

Table 2015.01A. Recommended concentrations for the calibration curve

Standard	As, µg/L	Cd, µg/L	Pb, µg/L	Hg, µg/L
0	0.00	0.00	0.000	0.00
1	0.01	0.01	0.005	0.01
2	0.02	0.02	0.010	0.05
3	0.10	0.10	0.050	0.10
4	0.50	0.50	0.250	0.50
5	5.00	5.00	2.500	2.00
6	20.00	20.00	10.000	5.00

rhodium (Rh), indium (In), and thulium (Tm) is recommended. Rh is analyzed in DRC mode for correction of the As signal. In addition, the presence of high levels of elements, such as carbon and chlorine, in samples can increase the effective ionization of the plasma and cause a higher response factor for arsenic in specific samples. This potential interference is addressed by the on-line addition of acetic acid (or another carbon source, such as methanol), which greatly increases the effective ionization of incompletely ionized analytes, and decreases the potential increase caused by sample characteristics. The internal standard solution should be prepared in 20% acetic acid.

(**f**) *Calibration standards.*—Fresh calibration standards should be prepared every day, or as needed.

(*1*) Dilute the multi-element stock standard solutions into 50 mL precleaned autosampler vials with 5% HNO_3 in such a manner as to create a calibration curve. The lowest calibration standard (STD 1) should be equal to or less than the limit of quantitation (LOQ) when recalculated in units specific to the reported sample results.

(*2*) *See* Table **2015.01A** for recommended concentrations for the calibration curve.

(**g**) *Initial calibration verification (ICV) solution.*—Made up from second source standards in order to verify the validity of the calibration curve.

(**h**) *Calibration solutions.*—Daily optimization, tuning, and dual detector calibration solutions, as needed, should be prepared and analyzed per the instrument manufacturer's suggestions.

(**i**) *Certified Reference Materials (CRMs).*—CRMs should preferably match the food matrix type being analyzed and contain the elements of interest at certified concentrations above the LOQ. Recommended reference materials include NIST SRM 1568a (Rice Flour), NIST SRM 1548a (Typical Diet), NRCC CRM DORM-3 (Dogfish Muscle), and NIST SRM 2976 (Mussel Tissue).

(**j**) *Spiking solution.*—50 mg/L Au and Lu in 5% (v/v) HNO_3. Prepared from single-element standards.

D. Contamination and Interferences

(**a**) Well-homogenized samples and small reproducible aliquots help minimize interferences.

(**b**) *Contamination.*—(*1*) Contamination of the samples during sample handling is a great risk. Extreme care should be taken to avoid this. Potential sources of contamination during sample handling include using metallic or metal-containing homogenization equipment, laboratory ware, containers, and sampling equipment.

(*2*) Contamination of samples by airborne particulate matter is a concern. Sample containers must remain closed as much as possible. Container lids should only be removed briefly and in a

clean environment during sample preservation and processing, so that exposure to an uncontrolled environment is minimized.

(**c**) *Laboratory.*—(*1*) All laboratory ware (including pipet tips, ICP-MS autosampler vials, sample containers, extraction apparatus, and reagent bottles) should be tested for the presence of the metals of interest. If necessary, the laboratory ware should be acid-cleaned, rinsed with DIW, and dried in a Class 100 laminar flow clean hood.

(*2*) All autosampler vials should be cleaned by storing them in 2% (v/v) HNO_3 overnight and then rinsed three times with DIW. Then dry vials in a clean hood before use. Glass volumetric flasks should be soaked in about 5% HNO_3 overnight prior to use.

(*3*) All reagents used for analysis and sample preparation should be tested for the presence of the metals of interest prior to use in the laboratory. Due to the ultra-low detection limits of the method, it is imperative that all the reagents and gases be as low as possible in the metals of interest. It is often required to test several different sources of reagents until an acceptable source has been found. Metals contamination can vary greatly from lot to lot, even when ordering from the same manufacturer.

(*4*) Keep the facility free from all sources of contamination for the metals of interest. Replace laminar flow clean hood HEPA filters with new filters on a regular basis, typically once a year, to reduce airborne contaminants. Metal corrosion of any part of the facility should be addressed and replaced. Every piece of apparatus that is directly or indirectly used in the processing of samples should be free from contamination for the metals of interest.

(**d**) *Elemental interferences.*—Interference sources that may inhibit the accurate collection of ICP-MS data for trace elements are addressed below.

(*1*) *Isobaric elemental interferences.*—Isotopes of different elements that form singly or doubly charged ions of the same *m/z* and cannot be resolved by the mass spectrometer. Data obtained with isobaric overlap must be corrected for that interference.

(*2*) *Abundance sensitivity.*—Occurs when part of an elemental peak overlaps an adjacent peak. This often occurs when measuring a small *m/z* peak next to a large *m/z* peak. The abundance sensitivity is affected by ion energy and quadrupole operating pressure. Proper optimization of the resolution during tuning will minimize the potential for abundance sensitivity interferences.

(*3*) *Isobaric polyatomic interferences.*—Caused by ions, composed of multiple atoms, which have the same *m/z* as the isotope of interest, and which cannot be resolved by the mass spectrometer. These ions are commonly formed in the plasma or the interface system from the support gases or sample components. The objective of IRT is to remove these interferences, making the use of correction factors unnecessary when analyzing an element in DRC mode. Elements not determined in DRC mode can be corrected by using correction equations in the ICP-MS software.

(**e**) *Physical interferences.*—(*1*) Physical interferences occur when there are differences in the response of the instrument from the calibration standards and the samples. Physical interferences are associated with the physical processes that govern the transport of sample into the plasma, sample conversion processes in the plasma, and the transmission of ions through the plasma-mass spectrometer interface.

(*2*) Physical interferences can be associated with the transfer of solution to the nebulizer at the point of nebulization, transport of aerosol to the plasma, or during excitation and ionization processes in the plasma. High levels of dissolved solids in a sample can result in physical interferences. Proper internal standardization

Table 2015.01B. Recommended isotopes for analysis

Element	Isotope, amu	Isotopic abundance, %	Potential interferences
Cd	111	13	MoO^+
	114	29	MoO^+, Sn^+
Hg	200	23	WO^+
	202	30	WO^+
Pb[a]	Sum of 206, 207, and 208	99	OsO^+

[a] Allowance for isotopic variability of lead isotopes.

(choosing internal standards that have analytical behavior similar to the associating elements) can compensate for many physical interferences.

(**f**) *Resolution of interferences.*—(*1*) For elements that are subject to isobaric or polyatomic interferences (such as As), it is advantageous to use the DRC mode of the instrument. This section specifically describes a method of using IRT for interference removal for As using a PerkinElmer DRC II and oxygen as the reaction gas. Other forms of IRT may also be appropriate.

(*a*) Arsenic, which is monoisotopic, has an *m/z* of 75 and is prone to interferences from many sources, most notably from chloride (Cl), which is common in many foods (e.g., salt). Argon (Ar), used in the ICP-MS plasma, forms a polyatomic interference with Cl at *m/z* 75 [$^{35}Cl + {}^{40}Ar = {}^{75}(ArCl)$].

(*b*) When arsenic reacts with the oxygen in the DRC cell, $^{75}As^{16}O$ is formed and measured at *m/z* 91, which is free of most interferences. The potential ^{91}Zr interference is monitored for in the following ways: ^{90}Zr and ^{94}Zr are monitored for in each analytical run, and if a significant Zr presence is detected, then $^{75}As^{16}O$ measured at *m/z* 91 is evaluated against the ^{75}As result. If a significant discrepancy is present, then samples may require analysis using alternative IRT, such as collision cell technology (helium mode).

(*c*) Instrument settings used (for PerkinElmer DRC II): DRC settings for $^{91}(AsO)$ and ^{103}Rh include an RPq value of 0.7 and a cell gas flow rate of 0.6 L/min. Cell conditions, especially cell gas flow rates, may be optimized for specific analyte/matrix combinations, as needed. In such cases, the optimized methods will often have slightly different RPq and cell gas flow values.

(*2*) For multi-isotopic elements, more than one isotope should be measured to monitor for potential interferences. For reporting purposes, the most appropriate isotope should be selected based on review of data for matrix interferences and based on the sensitivity (or relative abundance) of each isotope. The table below lists the recommended isotopes to measure. Low abundance isotopes are not recommended for this method as it is specifically applicable for ultra-low level concentrations (8–10 ppb LOQs). *See* Table **2015.01B**.

(**g**) *Memory effects.*—Minimize carryover of elements in a previous sample in the sample tubing, cones, torch, spray chamber, connections, and autosampler probe by rinsing the instrument with a reagent blank after samples high in metals concentrations are analyzed. Memory effects for Hg can be minimized through the addition of Au to all standard, samples, and quality control (QC) samples.

Table 2015.01C. Digestion program for Berghof Speedwave 4 microwave

Step	Temp., °C	Ramp, min	Hold, min
1	145	1	1
2	50	1	1
3	145	1	1
4	170	1	10
5	190	1	10

E. Sample Handling and Storage

(**a**) Food and beverage samples should be stored in their typical commercial storage conditions (either frozen, refrigerated, or at room temperature) until analysis. Samples should be analyzed within 6 months of preparation.

(**b**) If food or beverage samples are subsampled from their original storage containers, ensure that containers are free from contamination for the elements of concern.

F. Sample Preparation

(**a**) Weigh out sample aliquots (typically 0.25 g of as-received or wet sample) into microwave digestion vessels.

(**b**) Add 4 mL of concentrated HNO_3 and 1 mL of 30% hydrogen peroxide (H_2O_2) to each digestion vessel.

(**c**) Add 0.1 mL of the 50 mg/L Au + Lu solution to each digestion vessel.

(**d**) Cap the vessels securely (and insert into pressure jackets, if applicable). Place the vessels into the microwave system according to the manufacturer's instructions, and connect the appropriate temperature and/or pressure sensors.

(**e**) Samples are digested at a minimum temperature of 190°C for a minimum time of 10 min. Appropriate ramp times and cool down times should be included in the microwave program, depending on the sample type and model of microwave digestion system. Microwave digestion is achieved using temperature feedback control. Microwave digestion programs will vary depending on the type of microwave digestion system used. When using this mechanism for achieving performance-based digestion targets, the number of samples that may be simultaneously digested may vary. The number will depend on the power of the unit, the number of vessels, and the heat loss characteristics of the vessels. It is essential to ensure that all vessels reach at least 190°C and be held at this temperature for at least 10 min. The monitoring of one vessel as a control for the batch/carousel may not accurately reflect the temperature in the other vessels, especially if the samples vary in composition and/or sample mass. Temperature measurement and control will depend on the particular microwave digestion system.

(**1**) *Note*: a predigestion scheme for samples that react vigorously to the addition of the acid may be required.

(**2**) The method performance data presented in this method was produced using a Berghof Speedwave 4 microwave digestion

Table 2015.01D. Digestion program for CEM MARS 6 microwave

Step	Temp., °C	Ramp, min	Hold, min
1	190	20	10
2	Cool down	NA	10

Table 2015.01E. Digestion program for infant formula

Step	Temp., °C	Ramp, min	Hold, min
1	180	20	20
2	Cool down	NA	20
3	200	20	20
4	Cool down	NA	20

system, with the program listed in Table **2015.01C** (steps 1 and 2 are a predigestion step).

(**3**) Equivalent results were achieved using the program listed in Table **2015.01D** on a CEM MARS 6 microwave digestion system using the 40-position carousel and 55 mL Xpress digestion vessels.

(**4**) For infant formula samples, the program described in Table **2015.01E** has been shown to work effectively.

(**f**) Allow vessels to cool to room temperature and slowly open. Open the vessels carefully, as residual pressure may remain and digestate spray is possible. Pour the contents of each vessel into an acid-cleaned 50 mL HDPE centrifuge tube and dilute with DIW to a final volume of 20 mL.

(**g**) Digestates are diluted at least 4x prior to analysis with the 1% (v/v) HNO_3 diluent. When the metals concentration of a sample is unknown, the samples may be further diluted or analyzed using a total quantification method prior to being analyzed with a comprehensive quantitative method. This protects the instrument and the sample introduction system from potential contamination and damage.

(**h**) Food samples high in calcium carbonate ($CaCO_3$) will not fully digest. In such cases, the CRM can be used as a gauge for an appropriate digestion time.

(**i**) QC samples to be prepared with the batch (a group of samples and QC samples that are prepared together) include a minimum of three method blanks, duplicate for every 10 samples, matrix spike/matrix spike duplicate (MS/MSD) for every 10 samples, blank spike, and any matrix-relevant CRMs that are available.

G. Procedure

(**a**) *Instrument startup.*—(*1*) Instrument startup routine and initial checks should be performed per manufacturer recommendations.

(*2*) Ignite the plasma and start the peristaltic pump. Allow plasma and system to stabilize for at least 30 min.

(**b**) *Optimizations.*—(*1*) Perform an optimization of the sample introduction system (e.g., *X-Y* and *Z* optimizations) to ensure maximum sensitivity.

(*2*) Perform an instrument tuning or mass calibration routine whenever there is a need to modify the resolution for elements, or monthly (at a minimum), to ensure the instrument's quadrupole mass filtering performance is adequate. Measured masses should be ±0.1 amu of the actual mass value, and the resolution (measured peak width) should conform to manufacturer specifications.

(*3*) Optimize the nebulizer gas flow for best sensitivity while maintaining acceptable oxide and double-charged element formation ratios.

(*4*) Perform a daily check for instrument sensitivity, oxide formation ratios, double-charged element formation ratios, and background. If the performance check is not satisfactory, additional optimizations (a "full optimization") may be necessary.

Table 2015.01F. Summary of quality control samples

QC sample	Measure	Minimum frequency	Acceptance criteria	Corrective action
Calibration standards	Linearity of he calibration curve	Analyzed once per analytical day	Correlation coefficient ≥0.995, 1st standard ≤MRL, low standard recovery = 75–125%, all other standard recoveries = 80–120%	Reanalyze suspect calibra ion standard. If criteria still not met, then re-prepare standards and recalibrate the instrument.
Internal standards	Varia ion in sample properties between samples and standards	Each standard, blank, and sample is spiked with internal standard	60–125% recovery compared to calibration blank	If the responses of the internal standards in he following CCB are within the limit, rerun the sample at an additional 2x dilution. If not, then samples must be reanalyzed with a new calibration.
Lu digestion check spike	Assessment of potential loss during digestion	Added to every digested samples	Recovery ≥75%	Re-prepare the sample
Initial calibration verification (ICV)	Independent check of system performance	One following instrument calibration	Recovery = 90–110%	Correct problem prior to continuing analysis. Recalibrate if necessary.
Continuing calibration verification (CCV)	Accuracy	At beginning and end of analysis and one per 10 injections	Recovery = 85–115%	Halt analysis, correct problem, recalibrate, and reanalyze affected samples
Method blanks (MB)	Contamination from reagents, lab ware, etc.	Minimum of three per batch	Mean ≤ MRL; SD ≤ MDL or MBs <1/10th sample result	Determine and eliminate cause of contamination. Affected samples must be re-prepared and reanalyzed.
Method duplicates (MD)	Method precision within a given matrix	Minimum of one per 10 samples	RPD ≤ 30% or ±2x LOQ if results ≤5x LOQ	If RPD criteria not met, then sample may be re-prepared and reanalyzed, but this is not required. Sample matrix may be inhomogeneous. A post-digestion duplicate (PDD) can be analyzed to evaluate instrument precision.
Matrix spikes/matrix spike duplicates (MS/MSD)	Method accuracy and precision within a given matrix	Minimum of one per 10 samples	Recovery = 70–130% and RPD ≤ 30%	If RPD >30%, results must be qualified
Post-preparation spike (PS)	Check for matrix interference	When required (samples spiked too low/high, dilution test fails, etc.)	Recovery = 75–125%	Analyze samples using MSA or results flagged accordingly
Laboratory fortified blank (LFB) or blank spike (BS)	Method accuracy	Minimum of one per batch	Recovery = 75–125%	If LFB recovery is outside of the control limit, then batch must be re-prepared and reanalyzed
Certified Reference Material (CRM)	Method accuracy	Must be matrix-matched to samples; minimum of one per batch	Recovery = 75–125% unless limits set by CRM manufacturer are greater or element/CRM specific limits have been established	If CRM true value is ≥5x the LOQ and recovery is outside of the control limit, then batch must be re-prepared and reanalyzed

(**c**) *Internal standardization and calibration.*—(*1*) Following precalibration optimizations, prepare and analyze the calibration standards prepared as described in **C**(**e**).

(*2*) Use internal standardization in all analyses to correct for instrument drift and physical interferences. Refer to **D**(**e**)(*2*). Internal standards must be present in all samples, standards, and blanks at identical concentrations. Internal standards can be added using a second channel of the peristaltic pump to produce a responses that is clear of the pulse-to-analog detector interface.

(*3*) Multiple isotopes for some analytes may be measured, with only the most appropriate isotope (as determined by the analyst) being reported.

(*4*) Use IRT for the quantification of As using the Rh internal standard.

(**d**) *Sample analysis.*—(*1*) Create a method file for the ICP-MS.

(*2*) Enter sample and calibration curve information into the ICP-MS software.

(*3*) Calibrate the instrument and ensure the resulting standard recoveries and correlation coefficients meet specifications (**H**).

(*4*) Start the analysis of the samples.

(*5*) Immediately following the calibration, an initial calibration blank (ICB) should be analyzed. This demonstrates that there is no carryover of the analytes of interest and that the analytical system is free from contamination.

(*6*) Immediately following the ICB, an ICV should be analyzed. This standard must be prepared from a different source than the calibration standards.

(*7*) A minimum of three reagent/instrument blanks should be analyzed following the ICV. These instrument blanks can be used to assess the background and variability of the system.

(*8*) A continuing calibration verification (CCV) standard should be analyzed after every 10 injections and at the end of the run. The CCV standard should be a mid-range calibration standard.

(*9*) An instrument blank should be analyzed after each CCV (called a continuing calibration blank, or CCB) to demonstrate that there is no carryover and that the analytical system is free from contamination.

(*10*) Method of Standard Additions (MSA) calibration curves may be used any time matrix interferences are suspected.

(*11*) Post-preparation spikes (PS) should be prepared and analyzed whenever there is an issue with the MS recoveries.

(**e**) Export and process instrument data.

H. Quality Control

(**a**) The correlation coefficients of the weighted-linear calibration curves for each element must be ≥0.995 to proceed with sample analysis.

(**b**) The percent recovery of the ICV standard should be 90–110% for each element being determined.

(**c**) Perform instrument rinses after any samples suspected to be high in metals, and before any method blanks, to ensure baseline sensitivity has been achieved. Run these rinses between all samples in the batch to ensure a consistent sampling method.

(**d**) Each analytical or digestion batch must have at least three preparation (or method) blanks associated with it if method blank correction is to be performed. The blanks are treated the same as the samples and must go through all of the preparative steps. If method blank correction is being used, all of the samples in the batch should be corrected using the mean concentration of these blanks. The estimated method detection limit (EMDL) for the batch is equal to 3 times the standard deviation (SD) of these blanks.

(**e**) For every 10 samples (not including quality control samples), a matrix duplicate (MD) sample should be analyzed. This is a duplicate of a sample that is subject to all of the same preparation and analysis steps as the original sample. Generally, the relative percent difference (RPD) for the replicate should be ≤30% for all food samples if the sample concentrations are greater than 5 times the LOQ. RPD is calculated as shown below. An MSD may be substituted for the MD, with the same control limits.

$$RPD = 200 \times \frac{|S1 - S2|}{S1 + S2}$$

where S1 = concentration in the first sample and S2 = concentration in the duplicate.

(**f**) For every 10 samples (not including quality control samples), an MS and MSD should be performed. The percent recovery of the spikes should be 70–130% with an RPD ≤30% for all food samples.

(*1*) If the spike recovery is outside of the control limits, an MSA curve that has been prepared and analyzed may be used to correct for the matrix effect. Samples may be corrected by the slope of the MSA curve if the correlation coefficient of the MSA curve is ≥0.995.

(*a*) The MSA technique involves adding known amounts of standard to one or more aliquots of the processed sample solution. This technique attempts to compensate for a sample constituent that enhances or depresses the analyte signal, thus producing a different slope from that of the calibration standards. It will not correct for additive interferences which cause a baseline shift.

(*b*) The best MSA results can be obtained by using a series of standard additions. To equal volumes of the sample are added a series of standard solutions containing different known quantities of the analyte(s), and all solutions are diluted to the same final volume. For example, addition 1 should be prepared so that the resulting concentration is approximately 50% of the expected concentration of the native sample. Additions 2 and 3 should be prepared so that the concentrations are approximately 100% and 150%, respectively, of the expected native sample concentration. Determine the concentration of each solution and then plot on the vertical axis of a graph, with the concentrations of the known standards plotted on the horizontal axis. When the resulting line is extrapolated to zero absorbance, the point of interception of the abscissa is calculated MSA-corrected concentration of the analyte in the sample. A linear regression program may be used to obtain the intercept concentration.

(*c*) For results of the MSA technique to be valid, take into consideration the following limitations:

(*i*) The apparent concentrations from the calibration curve must be linear (0.995 or greater) over the concentration range of concern.

(*ii*) The effect of the interference should not vary as the ratio of analyte concentration to sample matrix changes, and the MSA curve should respond in a similar manner as the analyte.

(*2*) If the sample concentration levels are sufficiently high, the sample may be diluted to reduce the matrix effect. Samples should be diluted with the 1% (v/v) HNO_3 diluent. For example, to dilute a sample by a 10x dilution factor, pipette 1 mL of the digested sample into an autosampler vial, and add 9 mL of the 1% (v/v) HNO_3 diluent. MS/MSD sets should be performed at the same dilution factor as the native sample.

(*3*) Spike at 1–10 times the level of a historical sample of the same matrix type, or, if unknown, spike at 1–5 times a typical value for the matrix. Spiking levels should be no lower than 10 times the LOQ.

Table 2015.01G.   Method blank results and LOD/LOQ, µg/kg

Method blanks	^{91}(AsO)	^{111}Cd	^{114}Cd	Pb	^{200}Hg	^{202}Hg
MB-01	2.83	0.229	0 270	1.90	1.61	0.95
MB-02	1.48	−0.088	0 270	0.14	1.48	1.13
MB-03	1.80	0.007	0.115	0.13	0.76	0.25
MB-04	1.03	0.154	0 288	0.12	1.46	0.33
MB-05	1.43	0.010	0 259	1.84	1.28	0.27
MB-06	1.07	0.105	0 096	3.02	0.87	0.76
MB-07	2.31	−0.002	0 297	2.67	0.89	0.44
MB-08	1.20	0.285	0 200	4.24	0.55	0.28
MB-09	1.05	0.002	0.182	0.09	0.96	0.25
MB-10	2.12	0.047	0.150	0.19	0.71	0.02
MB-11	2.09	−0.145	0 226	0.12	0.64	0.57
MB-12	1.44	0.037	0.165	0.18	0.45	0.50
MB-13	0.70	−0.122	0.160	0.17	0.81	0.19
MB-14	1.12	−0.001	0 074	0.14	0.85	0.21
MB-15	2.33	0.097	0 207	0.11	0.18	0.17
MB-16	1.53	−0.117	0.146	0.16	1.33	1.09
MB-17	1.79	−0.070	0.180	0.03	3.46	2.19
MB-18	1.90	0.049	0.115	0.06	3.30	2.36
MB-19	1.18	0.043	0 224	0.39	4.01	2.78
MB-20	1.24	−0.060	0.199	0.07	0.99	0.56
MB-21	0.92	0.165	0.120	0.03	0.73	0.33
MB-22	1.69	0.005	0.186	0.09	0.60	0.25
MB-23	2.13	0.171	0.152	0.08	0.41	−0 23
SD	0.54	0.113	0 063	1.18	1.01	0.77
LOD	1.6	0.50^a	0.50^a	3.5	3.0	2 3
LOQ	3.3	1.60^a	1.60^a	7.1	6.0	4.6

^{a} Adjusted to conform to lowest calibration point.

(g) Percent recoveries of the CRMs should be 75–125% of their certified value.

(h) Percent recoveries of the CCV standards should be within 85–115%. Sample results may be CCV-corrected using the mean recovery of the bracketing CCVs. This should only be done after careful evaluation of the data. The instrument should show a trending drift of CCV recoveries and not just a few anomalous outliers.

(i) CCBs should be monitored for the effects of carryover and for possible system contamination. If carryover of the analyte at levels greater than 10 times the MDL is observed, the sample results may not be reportable.

(j) Absolute response of any one internal standard should not vary from the original response in the calibration blank by more than 60–125%. Some analytical samples, such as those containing concentrations of the internal standard and tissue digestates, can have a serious effect on the internal standard intensities, but this does not necessarily mean that the analytical system is out of control. In some situations, it is appropriate to reprocess the samples using a different internal standard monitored in the analysis. The data should be carefully evaluated before doing this.

(k) The recovery of the Lu that was spiked into the sample preparation prior to digestion should be evaluated to assess any potential loss of analyte during the process. The concentration of Lu in the sample preparation is 0.25 mg/L, and for samples diluted 4x at the instrument, this is equivalent to 62.5 µg/L at the instrument (if samples are diluted more than 4x, this must be taken into account). The Lu recovery should be no less than 75% of the original spiked concentration.

(l) Refer to Table 2015.01F for a summary of all recommended quality control samples, minimum frequency at which they are to be analyzed, acceptance criteria for each, and appropriate corrective action if the acceptance criteria are not met.

I. Method Performance

(a) Limit of detection (LOD) and LOQ were determined through the analysis of 23 method blanks (*see* Table 2015.01G). LOD was calculated as 3 times the SD of the results of the blanks, and LOQ was calculated as 2 times the value of the LOD, except where the resulting LOQ would be less than the lowest calibration point, in which case LOQ was elevated and set at the lowest calibration point and LOD was calculated as 1/3 of the LOQ. All LOQs achieved are ≤10 µg/kg for all food matrices and ≤8 µg/kg for liquid matrices, such as infant formula.

(b) Sample-specific LOQs for several matrices, based on LOQs determined by the default method, and adjusted for changes in sample mass for particular samples, are shown in Table 2015.01H. Values have been rounded up to the nearest part-per-billion.

(c) Numerous relevant CRMs were analyzed to establish method accuracy. Example percent recoveries are provided in Table 2015.01I (recoveries have been omitted for CRMs that do not provide a certified value or if the certified value is less than the LOQ).

Table 2015.01H.   Sample-specific LOQs

Sample	LOQ, µg/kg (as received)			
	As	Cd	Pb	Hg
Infant formula	2	1	4	3
Chocolate	4	2	8	6
Rice flour	4	2	8	6
Fruit juice	1	1	2	2

Table 2015.01I.   Recoveries for numerous relevant CRMs

Certified Reference Material	As, %	Cd, %	Pb, %	Hg, %
DOLT-4 Dogfish Liver	104	97	87	114
DORM-3 Fish Protein	105	109	94	114
DORM-4 Fish Protein	105	91	91	81
NIST 1548a Typical Diet	103	95	113	NA
NIST 1568a Rice Flour	98	99	NA	NA
NIST 1946 Lake Superior Fish Tissue	119	NA	NA	101
TORT-2 Lobster Hepatopancreas	109	104	95	116
TORT-3 Lobster Hepatopancreas	113	89	86	86

Table 2015.01J.   AOAC SMPR 2012.007 (ref. 1)

Concn range, µg/kg	Repeatability, %	Reproducibility, %	Recovery, %
LOQ–100	15	32	60–115
100–1000	11	16	80–115
>1000	7.3	8	80–115

(**d**) *Standard Method Performance Requirements* (AOAC SMPR® 2012.007; 1) for repeatability, reproducibility, and recovery for the method are shown in the Table **2015.01J**. *See* Appendix A (available on the *J. AOAC Int*. website as supplemental material, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac) for detailed method performance information supporting acceptance of the method.

(**e**) *See* Appendix A for detailed method performance information supporting acceptance of the method. Method validation samples were prepared and analyzed for all applicable matrices. In general, all SMPR criteria were met for As, Cd, Hg, and Pb in the matrices apple juice, infant formula, cocoa powder, and rice flour.

References: (1) AOAC SMPR 2012.007
           J. AOAC Int. **96**, 704(2013)
           DOI: 10.5740/jaoac.int.2012.007

           J. AOAC Int. **98**, 1113(2015)
           DOI: 10.5740/jaoac.int.2015.01

Posted: September 9, 2015

Method: **Determination of Heavy Metals by ICP-MS**

Reference: **AOAC Method 2015.01**

Approved: (b) (4)   Date: 4/25/19

1.   **Purpose**
       This method is to describe the steps for preparation of samples and standards to perform quantitative determination of metal impurities by microwave digestion and analysis by ICP-MS.

2.   **Scope**
       This method is applicable for the detection of metal impurities by ICP-MS. This method is suitable for a range of elements to be quantified; however, the elements of primary concern are arsenic, cadmium, lead and mercury.

3.   **Background**
       This method should be used by analysts familiar with trace element analysis and ICP-MS.

4.   **Responsibilities**

   4.1 Laboratory Co-Director authorized to assign and approve subject analysis is responsible for

   - Approving Method Folder content
   - Assuring the sample is fit for use
   - Resolving analytical issues and deficiencies with subject analysis

   4.2 Section Supervisor authorized to conduct subject analysis is responsible for

   - Approving assigned analyst work
   - Assuring the Method Folder is up to date including content and appendices
   - Discussing any deviations with the Laboratory Co-Director

   4.3 Analyst authorized to conduct this analysis is responsible for

   - Reviewing Method Folder instructions prior to initiating analysis, especially for matrix applicability
   - Analyzing the sample according to documented instructions
   - Assessing method and instrument performance both real time and at reporting
   - Addressing any deviation from instructions or specifications with the Section Supervisor
   - Updating Method Folder performance data

5.0 References

   5.1 Method

   - AOAC INTERNATIONAL. Official Methods of Analysis, 20th ed., Method 2015.01 – Heavy Metals in Food – Inductively Coupled Plasma-Mass Spectrometry.
   - FDA EAM (Elemental Analysis Manual) 4.7 Vesrion 1.1 (March 2015), P. Gray, W. Midak, J. Cheng – "Inductively Coupled Plasma-Mass Spectrometric Determination of Arsenic,

Cadmium, chromium, Lead, Mercury and Other Elements in Food Using Microwave Assisted Digestion"
- Perkin Elmer – "Determination of Elemental Impurities in Cannabis and Related Materials by Indirect Closed-Vessel Microwave Digestion and ICP-MS Analysis"

5.2 Instrumentation

- Perkin Elmer NexION 1000/2000 ICP-MS

6.0 Method Folder

6.1 Instrumentation

The analyst authorized to perform this test method must be deemed knowledgeable in the operation of the instrumentation cited in **5.2 Instrumentation**

6.2 Safety

This method does not address all safety issues associated with its use. The analyst must establish appropriate safety and health practice prior to initiating analysis. The analyst must be familiar with (b) (4) hazardous waste plan.

Reagents should be regarded as potential health hazards and exposure to these compounds should be limited.

6.3 Definitions

Analytical sample – sample, prepared by the laboratory (by homogenization, grinding, blending, etc.), from which analytical portions (aliquots) are removed for analysis.

Analytical portion – quantity of material removed from the analytical sample.

Analytical solution – solution prepared by decomposing an analytical portion and diluting to volume.

Batch – a group of analytical portions processed in a continuous sequence under relatively stable conditions. Specifically:

- Method is constant
- Instrument and its conditions (i.e. pertinent operating parameters) are constant
- Standardization is constant

Dilution Factor (DF) – factor by which concentration in a diluted solution (e.g. diluted analytical solution) is multiplied to obtain concentration in the initial solution (e.g. analytical solution).

Method Blank (MBK) – solution that is prepared using all reagents and exposed to all laboratory ware, apparatus, equipment, digestion process and analyses in the same manner as if it were an analytical portion being analyzed without the sample. The MBK is analyzed to ensure analytes have not significantly been added to the analytical portion from materials and laboratory environment.

Reagent Blank (RB) – solution that is prepared using the same labware, acids, and dilution as calibration standards, prepare a solution as if it were a calibration standard without added sample.

Reference material (RM) – food related materials developed for analytical quality control, which have reference value concentration for the element of interest.

Independent calibration verification (ICV) – solution of method analytes of known concentration obtained from a source external to the laboratory and different from the source used for instrument standardization. The ICV is used to ensure a valid standardization and to check laboratory performance.

Continuous calibration verification (CCV) – verification of one of the calibration standard points. It is used to verify the calibration accuracy during the analysis of the analytical batch.

Matrix Spike (SP) – analytical portion fortified (spiking) with the analyte before digestion. Measurement of the final concentration of the analyte is made according to the analytical method. The purpose of the spike is to determine if the preparation procedure or sample matrix contribute bias to the results.

Blank Spike (BS) – solution that is spiked with known concentration analytes and prepared using the same labware, acids, dilutions and exposed to the same digestion process as the Method Blank. The purpose is to determine the spiked analyte recoveries to determine the accuracy.

Internal Standards Solution (ISS) – non analyte solution that is added to all calibration standards, quality control and analyzed samples, which uses the isotope ratio to correct for the instrument drift and matrix interferences.

Stock standard solution – a solution containing a high concentration of the analyte purchased from a reputable commercial source. Stock standard solutions are used to prepare standard solutions and other needed analyte solutions.

Intermediate standard solution – a solution containing one or more analytes prepared in the laboratory by diluting an aliquot of stock solution.

Standard solution – a solution prepared from the dilution of stock standard or intermediate standard solutions. Standard solutions are used to standardize instrument response (absorbance) to analyte concentration.

Analytical solution detection limit (ASDL) – an estimate of the lowest concentration of the analyte element in a MBK according to the statistics of hypothesis with a 95% confidence.

Limit of detection (LOD) – an estimate of the element concentration a method can detect in an analytical portion according to the statistics of hypothesis testing with a 95% confidence.

Limit of Quantitation (LOQ) – the minimum concentration of an analyte in a specific matrix that can be reliably quantified while also meeting predefined goals for bias and imprecision.

7.0 Method Work Level Instructions

7.1 Equipment and materials

(a) Analytical Balance – capable of weighing to the nearest 0.001 gram.
(b) Digestion vials – disposable glass tubes
(c) Microwave Digestor – Milestone UltraWave
(d) ICP-MS – Perkin Elmer

7.2 Reagents and Standards

All reagents may contain impurities that may affect the integrity of the analytical results. Due to the high sensitivity of the ICP-MS, high-purity reagents, water, acids, glassware and sample tubes that are suitable for trace metal analysis must be used at all time.

(a) 100 mg/L (ppm) Gold (Au) Stock Standard
(b) 1000 mg/L (ppm) Arsenic (As) Stock Standard
(c) 1000 mg/L (ppm) Cadmium (Cd) Stock Standard
(d) 1000 mg/L (ppm) Lead (Pb) Stock Standard
(e) 1000 mg/L (ppm) Mercury (Hg) Stock Standard
(f) Nitric Acid (HNO_3) – Concentrated (sp gr 1.41), trace metal grade
(g) Hydrochloric Acid (HCl) – Concentrated, trace element grade
(h) Internal Standard Solution – 50 mg/L Germanium (Ge), 20 mg/L Gallium (Ga), 1 mg/L Indium (In), 1 mg/L Terbium (Tb)
(i) Deionized water (DI H_2O)

7.2.1 Working solutions

Please always use safety precautions when preparing solutions. Always add acid to water! Shake each solution after all the reagents are combined.

(b) (4)

(b) (4)

(b) (4)

7.3 Test Sample Treatment

Milestone UltraWave microwave is used to digest in order to prepare the analytical batch.

7.3.1   Sample Preparation:

(b) (4)

(b) (4)
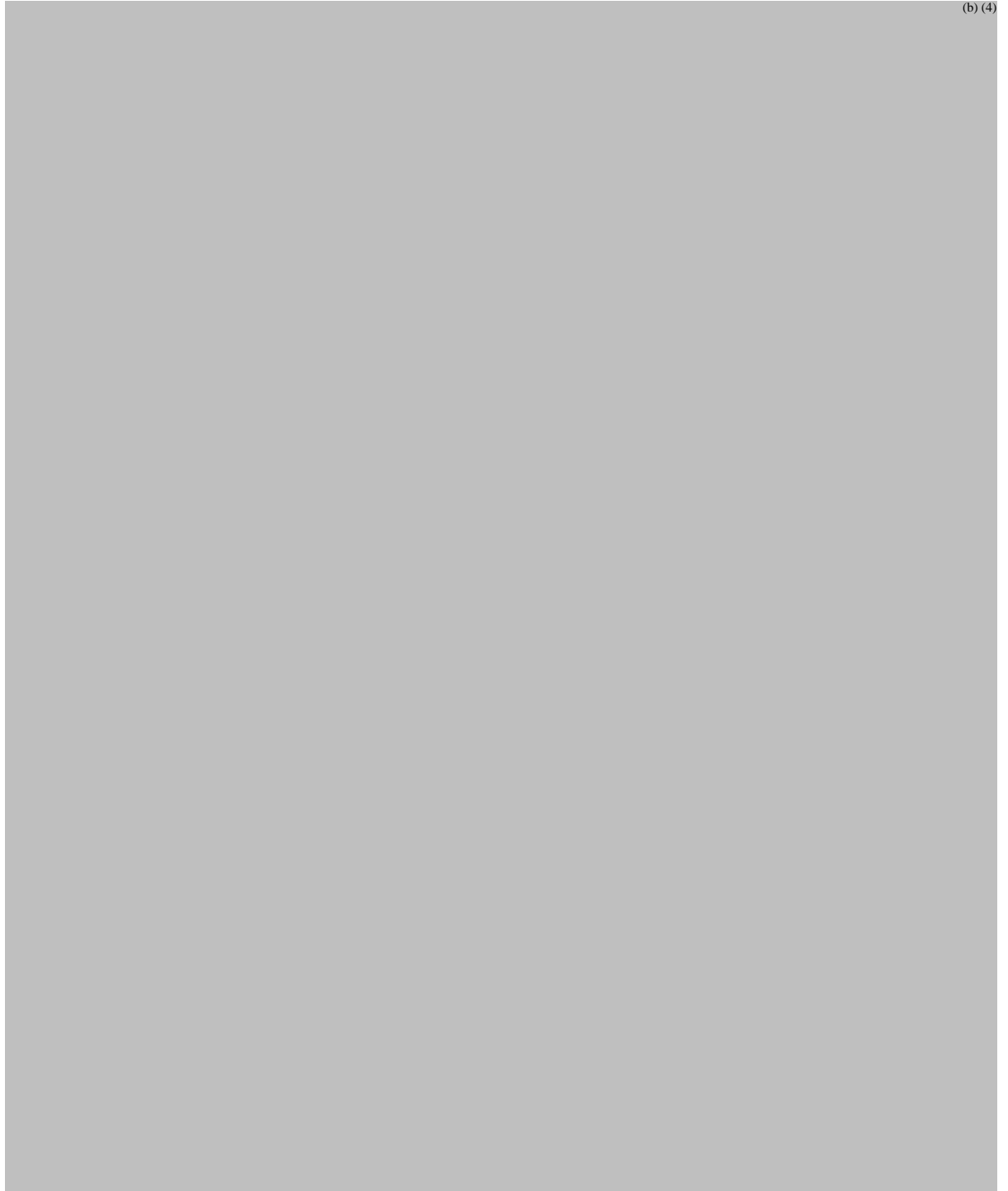
7.4 Instrumentation Set up

7.4.1   Start-Up Procedure:

(b) (4)

7.4.2   Tuning:

(b) (4)

7.4.3   Running Samples:

(b) (4)

(b) (4)

(b) (4)

7.4.4 While Running:

(b) (4)

7.4.5 Data Processing:

(b) (4)

Appendix A - Calibration Concentrations

Calibrations (b) (4)

AOAC Official Method 2013.10
Listeria species in a Variety of Foods and Environmental Surfaces
VIDAS® UP *Listeria* (LPT) Method
First Action 2013
Final Action 2016

[Applicable to detection of *Listeria* in deli ham (25 and 125 g), pepperoni (25 g), beef hot dogs (25 g), chicken nuggets (25 g), chicken liver pâté (25 g), ground beef (125 g), deli turkey (125 g), cooked shrimp (25 g), smoked salmon (25 g), whole cantaloupe melon, bagged mixed salad (25 g), peanut butter (25 g), black pepper (25 g), vanilla ice cream (25 g), queso fresco (25 and 125 g), stainless steel, plastic, ceramic and concrete environmental surfaces.]

See Tables **2013.10A** and **B** for a summary of results of the collaborative study. *See* supplemental data, Tables 2A–D, for detailed results of the collaborative study on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac.

Caution: *Listeria monocytogenes* is of particular concern for pregnant women, the aged, and the infirmed. It is recommended that these concerned groups avoid handling this organism. Dispose of all reagents and other contaminated materials by acceptable procedures for potentially biohazardous materials. Some reagents in the kit contain 1 g/L concentrations of sodium azide. Check local regulations prior to disposal. Disposal of these reagents into sinks with copper or lead plumbing should be followed immediately with large quantities of water to prevent potential hazards. This kit contains products of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not totally guarantee the absence of transmissible pathogenic agents. It is, therefore, recommended that these products be treated as potentially infectious and handled observing the usual safety precautions (do not ingest or inhale).

A. Principle

VIDAS® UP *Listeria* (LPT) method is for use on the automated VIDAS instrument for the detection of *Listeria* antigens using the enzyme-linked fluorescent assay (ELFA) method. The assay also incorporates phage proteins allowing an increase in sensitivity and specificity compared to traditional immunoassay. The Solid Phase Receptacle (SPR®) serves as the solid phase as well as the pipetting device. The interior of the SPR is coated with proteins specific for *Listeria* receptors. Reagents for the assay are ready-to-use and predispensed in the sealed reagent strips. All of the assay steps are performed automatically by the instrument. The reaction medium is cycled in and out of the SPR several times. An aliquot of enrichment broth is dispensed into the reagent strip. The *Listeria* receptors present will bind to the interior of the SPR. Unbound components are eliminated during the washing steps. The proteins conjugated to the alkaline phosphatase are cycled in and out of the SPR and will bind to any *Listeria* receptors, which are themselves bound to the SPR wall. A final wash step removes unbound conjugate. During the final detection step, the substrate (4-methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methyl-umbelliferone), the fluorescence of which is measured at 450 nm. At the end of the assay, results are automatically analyzed by the instrument, which calculates a test value for each sample. This value is then compared to internal references (thresholds) and each result is interpreted as positive or negative.

B. Apparatus and Reagents

Items (**a**)–(**h**) are available as the VIDAS UP *Listeria* (LPT) assay kit from bioMérieux (Hazelwood, MO, USA).

(**a**) *VIDAS or miniVIDAS automated immunoassay system*.

(**b**) *LPT reagent strips.*—Sixty polypropylene strips of 10 wells, each strip covered with a foil seal and label. The 10 wells contain the reagents shown in Table **2013.10C**.

(**c**) *SPR.*—Sixty SPRs coated with proteins specific for *Listeria* receptors.

(**d**) *Standard.*—One vial (1 × 6 mL). Ready-to-use. Contains purified and inactivated *Listeria* receptors + preservative + protein stabilizer.

(**e**) *Positive control solution.*—1 × 6 mL. Contains purified and inactivated *Listeria monocytogenes* antigen + preservative + protein stabilizer.

(**f**) *Negative control solution.*—1×6 mL. Contains Tris-buffered saline (TBS; 150 mmol/l) – Tween pH 7.6 + preservative.

(**g**) *Master Lot Entry (MLE) card.*—One card providing specifications for the factory master data required to calibrate the test: To read the MLE data, please refer to the Operator's Manual.

(**h**) *Package insert*.

(**i**) *Disposable pipet.*—To dispense appropriate volumes.

(**j**) *VIDAS Heat and Go.*—Available from bioMérieux, Inc.

(**k**) *Water bath.*—95–100°C, or equivalent.

(**l**) *Bag with filter.*

(**m**) *Smasher™ Blender/Homogenizer.*—Available from bioMérieux, Inc., or equivalent.

(**n**) *LPT broth.*—bioMérieux, Inc.

(**o**) *Incubators.*—Capable of maintaining 30±1°C and 35±1°C.

(**p**) *Diagnostic reagents.*—Necessary for culture confirmation of assays.

(**q**) *ALOA chromogenic agar.*—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**r**) *Tryptic Soy Agar with yeast additive*.

C. General Instructions

(**a**) Components of the kit are intended for use as integral unit. Do not mix reagents or disposables of different lot numbers.

(**b**) Store VIDAS LPT kits at 2–8°C.

(**c**) Do not freeze reagents.

(**d**) Bring reagents to room temperature before inserting them into the VIDAS instrument.

(**e**) Standard, controls, and heated test portions are mixed well before using.

(**f**) Include one positive and one negative control with each group of tests.

(**g**) Return unused components to 2–8°C immediately after use.

(**h**) *See* safety precautions in the VIDAS LPT package insert (Warnings and Precautions and Waste Disposal).

(**i**) *See* Centers for Disease Control recommendations in handling pathogens. http:/www.cdc.gov/biosafety/publications/bmb15/index htm/

Table 2013.10A. Summary of results for the detection of *Listeria* spp. in queso fresco (25 g)[a]

	VIDAS LPT with OXA			VIDAS LPT with ALOA		
	Inoculation level					
	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/ total No. samples analyzed	1/156	80/156	156/156	1/156	80/156	156/156
Candidate presumptive POD (CP)	0.01	0.51	1.00	0.01	0.51	1.00
	(0.01, 0.04)	(0.43, 0.59)	(0.98, 1.00)	(0.01, 0.04)	(0.43, 0.59)	(0.98, 1.00)
s_r[b]	0.08	0.51	0.00	0.08	0.51	0.00
	(0.07, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.07, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L[c]	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.03)	(0.00, 0.13)	(0.00, 0.15)	(0.00, 0.03)	(0.00, 0.13)	(0.00, 0.15)
s_R[d]	0.08	0.51	0.00	0.08	0.51	0.00
	(0.07, 0.13)	(0.46, 0.52)	(0.00, 0.21)	(0.07, 0.13)	(0.46, 0.52)	(0.00, 0.21)
P value[e]	0.4395	0.9210	1.0000	0.4395	0.9210	1.0000
Candidate confirmed positive/ total No. samples analyzed	0/156	78/156	156/156	0/156	78/156	156/156
Candidate confirmed POD (CC)	0.00	0.50	1.00	0.00	0.50	1.00
	(0.00, 0.02)	(0.42, 0.58)	(0.98, 1.00)	(0.00, 0.02)	(0.42, 0.58)	(0.98, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.15)	(0.00, 0.14)	(0.00, 0.15)	(0.00, 0.15)	(0.00, 0.14)	(0.00, 0.15)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.21)	(0.46, 0.52)	(0.00, 0.21)	(0.00, 0.21)	(0.46, 0.52)	(0.00, 0.21)
P value	1.0000	0.9161	1.0000	1.0000	0.9161	1.0000
Positive reference samples/ total No. samples analyzed	0/156	76/156	156/156	0/156	76/156	156/156
Reference POD	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.02)	(0.41, 0.57)	(0.98, 1.00)	(0.00, 0.02)	(0.41, 0.57)	(0.98, 1.00)
s_r	0.00	0.52	0.00	0.00	0.52	0.00
	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.15)	(0.00, 0.10)	(0.00, 0.15)	(0.00, 0.15)	(0.00, 0.10)	(0.00, 0.15)
s_R	0.00	0.52	0.00	0.00	0.52	0.00
	(0.00, 0.21)	(0.47, 0.52)	(0.00, 0.21)	(0.00, 0.21)	(0.47, 0.52)	(0.00, 0.21)
P value	1.0000	0.9937	1.0000	1.0000	0.9937	1.0000
dLPOD (candidate vs reference)	0.00	0.01	0.00	0.00	0.01	0.00
	(−0.02, 0.02)	(−0.10, 0.13)	(−0.02, 0.02)	(−0.02, 0.02)	(−0.10, 0.13)	(−0.02, 0.02)
dLPOD (candidate presumptive vs candidate confirmed)	0.01	0.01	0.00	0.01	0.01	0.00
	(−0.02, 0.04)	(−0.10, 0.13)	(−0.02, 0.02)	(−0.02, 0.04)	(−0.10, 0.13)	(−0.02, 0.02)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P* value = Homogeneity test of laboratory PODs.

Table 2013.10B. Summary of results for the detection of *Listeria* spp. in queso fresco (125 g)[a]

	VIDAS LPT with OXA			VIDAS LPT with ALOA		
	Inoculation level					
	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/ total No. of samples analyzed	0/144	70/144	144/144	0/144	70/144	144/144
Candidate presumptive POD (CP)	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)
s_r[b]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L[c]	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R[d]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value[e]	1.0000	0.9730	1.0000	1.0000	0.9730	1.0000
Candidate confirmed positive/ total No. of samples analyzed	0/144	70/144	144/144	0/144	70/144	144/144
Candidate confirmed POD (CC)	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value	1.0000	0.9730	1.0000	1.0000	0.9730	1.0000
Positive reference samples/ total No. of samples analyzed	0/144	69/144	144/144	0/144	69/144	144/144
Reference POD	0.00	0.48	1.00	0.00	0.48	1.00
	(0.00, 0.03)	(0.39, 0.56)	(0.97, 1.00)	(0.00, 0.03)	(0.39, 0.56)	(0.97, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value	1.0000	0.9672	1.0000	1.0000	0.9672	1.0000
dLPOD (C vs R)	0.00	0.01	0.00	0.00	0.01	0.00
	(−0.03, 0.03)	(−0.10, 0.13)	(−0.03, 0.03)	(−0.03, 0.03)	(−0.10, 0.13)	(−0.03, 0.03)
dLPOD (CP vs CC)	0.00	0.00	0.00	0.00	0.00	0.00
	(−0.03, 0.03)	(−0.12, 0.12)	(−0.03, 0.03)	(−0.03, 0.03)	(−0.12, 0.12)	(−0.03, 0.03)

[a]   Results include 95% confidence intervals.

[b]   Repeatability standard deviation.

[c]   Among-laboratory standard devia ion.

[d]   Reproducibility standard deviation.

[e]   *P* value = Homogeneity test of laboratory PODs.

Table 2013.10C. Reagents included in 10-well reagent strip

Wells	Reagents (LPT)
1	Sample well: 0.5 mL of enrichment broth, standard or control
2	Prewash solution (400 µL): TRIS-NaCl (150 mmol/L) - Tween pH 7.6 + preservative
3–5, 7–9	Wash buffer (600 µL): TRIS-NaCl (150 mmol/L) - Tween pH 7.6 + preservative
6	Conjugate (400 µL): alkaline phosphatase-labeled proteins specific for *Listeria* receptors + preservative
10	Reading cuvette with substrate (300 µL): 4-methyl-umbelliferyl phosphate (0.6 mmol/L) + diethanolamine[a] (DEA) (0.62 mol/L or 6.6%, pH 9.2) + preservative

[a] Irritant reagent: *See* VIDAS LPT package insert for more information.

D. Preparation of Test Suspension

(**a**) *Pre-enrichment.*—Pre-enrich test portion using filter Stomacher type bags to initiate growth of *Listeria.* For 25 g test portions, add 225 mL prewarmed (18–25°C) LPT broth to each test portion and homogenize thoroughly for 2 min. For cantaloupe melons, soak entire melon in approximately 1 L prewarmed (18–25°C) LPT broth. For 125 g test portions, add 375 mL prewarmed (18–25°C) LPT broth to each test portion and homogenize thoroughly for 2 min.

(**b**) *Test portions.—(1) 25 g test portions/cantaloupe melons rinses.*—After homogenization, incubate for 26–30 h at 30 ± 1°C.

(2) 125 g test portions.—After homogenization, incubate for 24–30 h at 30 ± 1°C.

From the primary enrichment broth, transfer a 1 mL aliquot into 10 mL prewarmed (18–25°C) LPT broth and incubate for 22–26 h at 30 ± 1°C.

(**c**) After incubation, homogenize samples manually. Follow appropriate instructions based on heating method.

(*1*) *Boiling.*—Transfer 2–3 mL of the enrichment broth into a tube. Seal the tube. Heat in a water bath for 5 ± 1 min at 95–100°C. Cool the tube. Mix the boiled broth and transfer 0.5 mL into the sample well of the VIDAS LPT reagent strip. Perform the VIDAS test.

(2) Heat and Go.—Transfer 0.5 mL of the enrichment broth into the sample well of the VIDAS LPT reagent strip. Heat for 5 ± 1 min (*see* VIDAS Heat and Go User's Manual). Remove the strip and allow to cool for 10 min prior to test initiation. Perform the VIDAS test.

E. Enzyme Immunoassay

(**a**) Enter factory master calibration curve data into the instrument using the MLE card.

(**b**) Remove the kit reagents and materials from refrigerated storage and let them to come to room temperature for at least 30 min.

(**c**) Use one VIDAS LPT reagent strip and one VIDAS LPT SPR for each sample, control, or standard to be tested. Reseal the storage pouch after removing the required number of SPRs.

(**d**) Enter the appropriate assay information to create a work list. Enter the test code by typing or selecting "LPT," and number of tests to be run. If the standard is to be tested, identify the standard by "S1" and test in duplicate. If the positive control is to be tested, identify it by "C1." If the negative control is to be tested, identify it by "C2."

Table 2013.10D. Interpretation of test

Test value threshold	Interpretation
<0.05	Negative
≥0.05	Positive

Note: The standard must be tested upon receipt of a new lot of reagents and then every 14 days. The relative fluorescence value (RFV) of the standard must fall within the set range provided with the kit.

(**e**) Load the LPT reagents strips and SPRs into the positions that correspond to the VIDAS section indicated by the work list. Verify that the color labels with the assay code on the SPRs and reagent strips match.

(**f**) Initiate the assay processing as directed in the VIDAS operator's manual.

(**g**) After the assay is completed, remove the SPRs and reagent strips from the instrument and dispose of properly.

F. Results and Interpretation

The results are analyzed automatically by the VIDAS system. A report is printed which records the type of test performed, the test sample identification, the date and time, the lot number and expiration date of the reagent kit being used, and each sample's RFV, test value, and interpreted result (positive or negative). Fluorescence is measured twice in the reagent strip's reading cuvette for each sample tested. The first reading is a background reading of the substrate cuvette before the SPR is introduced into the substrate. The second reading is taken after incubating the substrate with the enzyme remaining on the interior of the SPR. The test value is calculated by the instrument and is equal to the difference between the background reading and the final reading. The calculation appears on the result sheet. A "negative" result has a test value less than the threshold (0.05) and indicates that the sample does not contain *Listeria* spp. or contains *Listeria* spp. at a concentration below the detection limit. A "positive" result has a test value equal to or greater than the threshold (≥0.05) and indicates that the sample may be contaminated with *Listeria* spp. If the background reading is above a predetermined cutoff, then the result is reported as invalid (Table **2013.10D**).

G. Confirmation

All positive VIDAS LPT results must be culturally confirmed. Confirmation should be performed using the nonheated enrichment broth stored between 2–8°C and should be initiated within 72 h following the end of incubation (AFNOR Certificate No. BIO 12/33-05/12). Presumptive positive results may be confirmed by isolating on selective agar plates such as ALOA or on the appropriate reference method selective agar plates. Typical or suspect colonies from each plate are confirmed as described in appropriate reference method. As an alternative to the conventional confirmation for *Listeria*, **2012.02** VITEK 2 GP Biochemical Identification or API *Listeria* biochemical kits may be used for presumptive generic identification of foodborne *Listeria*.

Posted: May 2014, February 2016

AOAC Official Method 2013.01
Salmonella in a Variety of Foods
VIDAS® UP *Salmonella* (SPT) Method
First Action 2013
Final Action 2016

[Applicable to detection of *Salmonella* in raw ground beef (25 and 375 g), processed American cheese (25 g), deli roast beef (25 g), liquid egg (25 g), peanut butter (25 g), vanilla ice cream (25 g), cooked shrimp (25 g), raw cod (25 g), bagged lettuce (25 and 375 g), dark chocolate (375 g), powdered eggs (25 g), instant nonfat dry milk (25 and 375 g), ground black pepper (25 g), dry dog food (375 g), raw ground turkey (375 g), almonds (375 g), chicken carcass rinsates (30 mL), and stainless steel, plastic, and ceramic environmental surfaces.]

See Tables **2013.01A** and **B** for a summary of results of the interlaboratory study. For detailed results of the interlaboratory study, *see* Tables A–F in Appendix 1 on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac).

A. Principle

The VIDAS SPT method is for use on the automated VIDAS instrument for the detection of *Salmonella* receptors using the enzyme-linked fluorescent assay. The solid-phase receptacle (SPR) serves as the solid phase, as well as the pipetting device. The interior of the SPR is coated with proteins specific for *Salmonella* receptors. Reagents for the assay are ready-to-use and predispensed in the sealed reagent strips. The instrument performs all the assay steps automatically. The reaction medium is cycled in and out of the SPR several times. An aliquot of enrichment broth is dispensed into the reagent strip. The *Salmonella* receptors present will bind to the interior of the SPR. Unbound components are eliminated during the washing steps. The proteins conjugated to the alkaline phosphatase are cycled in and out of the SPR and will bind to any *Salmonella* receptors, which are themselves bound to the SPR wall. A final wash step removes unbound conjugate. During the final detection step, the substrate (4-methylumbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methylumbelliferone), the fluorescence of which is measured at 450 nm. At the end of the assay, results are automatically analyzed by the instrument which calculates a test value for each sample. This value is then compared to internal references (thresholds) and each result is interpreted as positive or negative.

B. Apparatus and Reagents

Items (**a**)–(**h**) are available as the VIDAS SPT assay kit from bioMérieux Inc., Hazelwood, MO.

(**a**) *VIDAS or miniVIDAS automated immunoassay system.*

(**b**) *SPT reagent strips.*—60 polypropylene strips of 10 wells, each strip covered with a foil seal and label. The 10 wells contain the reagents in Table **2013.01C**.

(**c**) *SPR.*—60 SPRs coated with proteins specific for *Salmonella* receptors.

(**d**) *Standard.*—One vial (6 mL). Contains purified and inactivated *Salmonella* receptors + preservative + protein stabilizer.

(**e**) *Positive control solution.*—One vial (6 mL). Contains purified and inactivated *Salmonella* receptors + preservative + protein stabilizer.

(**f**) *Negative control solution.*—One vial (6 mL). Contains Tris-buffered saline (150 mmol/L)–Tween pH 7.6 + preservative.

(**g**) *Master lot entry (MLE) card.*—One card providing specifications for the factory master data required to calibrate the test.

(**h**) *Package insert*.

(**i**) *Disposable pipet to dispense appropriate volumes.*

(**j**) *VIDAS Heat and Go.*—Available from bioMérieux, Inc.

(**k**) *Water bath (95–100°C) or equivalent system.*

(**l**) *Stomacher®-type bag with filter.*

(**m**) *Stomacher.*—Stomacher Lab Blender 400, available from Seward Medical (London, UK); Smasher, bioMérieux, Inc., or equivalent.

(**n**) *BPW.*—Available from bioMérieux, Inc.

(**o**) *Salmonella supplement.*—Available from bioMérieux, Inc.

(**p**) *Incubators.*—Capable of maintaining 42±1°C and 35±1°C.

(**q**) *Diagnostic reagents.*—Necessary for culture confirmation of assays. *See* **967.27** (*see* 17.9.03).

(**r**) *IBISA chromogenic agar.*—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**s**) *ASAP chromogenic agar.*—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**t**) *Vancomycin.*—Available from bioMérieux, Inc.

C. General Instructions

(**a**) Components of the kit are intended for use as integral unit. Do not mix reagents or disposables of different lot numbers.

(**b**) Store VIDAS SPT kits at 2–8°C.

(**c**) Do not freeze reagents.

(**d**) Bring reagents to room temperature before inserting them into the VIDAS instrument.

(**e**) Mix standard, controls, and heated test portions well before using.

(**f**) Include one positive and one negative control with each group of tests.

(**g**) Return unused components to 2–8°C immediately after use.

(**h**) *See* safety precautions in the VIDAS SPT package insert (refer to the following sections in the package insert: Warnings and Precautions and Waste Disposal).

D. Preparation of Test Suspension

(**a**) *Pre-enrichment.*—Pre-enrich test portion in BPW using filter Stomacher bags to initiate growth of *Salmonella.* For 25 g test portions, add 225 mL BPW to each test portion and homogenize thoroughly for 2 min. For 375 g test portions, prewarm BPW to 42±1°C, add 1125 mL to each test portion, and homogenize thoroughly for 2 min.

(**b**) After homogenization add *Salmonella* supplement to each test portion. For 25 g test portions, add 1 mL of *Salmonella* supplement, mix samples manually, and incubate for 18–24 h at 42±1°C. For 375 g test portions, add 5 mL of *Salmonella* supplement, mix samples manually, and incubate for 22–26 h at 42±1°C.

(**c**) After incubation, homogenize samples manually. If a water bath is used, transfer 2–3 mL enrichment broth into a tube. Seal the tube. Heat for 5±1 min at 95–100°C. Cool the tube. Mix the boiled broth and transfer 0.5 mL into the sample well of the VIDAS SPT reagent strip. If the VIDAS Heat and Go is used, transfer 0.5 mL of the enrichment broth into the sample well of the VIDAS SPT reagent strip. Heat for 5±1 min (*see* VIDAS Heat and Go User's

Table 2013.01A. Summary of results for the detection of *Salmonella* spp. in raw ground beef (25 g)

Method[a] / Inoculation level	V DAS SPT with traditional confirmation on BGSA and XLT4			VIDAS SPT with traditional confirmation on IBISA and ASAP[b]			VIDAS SPT with alternative confirmation on IBISA and ASAP[c]		
	Uninoculated	Low	High	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total samples analyzed	0/144	144/144	144/144	0/144	144/144	144/144	0/144	144/144	144/144
Candidate presumptive POD (CP)	0.00 (0.00, +0.03)	1.00 (+0.97, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	1.00 (+0.97, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	1.00 (+0.97, +1.00)	1.00 (+0.97, +1.00)
s_r[d]	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)
s_L[e]	0.00 (0.00 +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)
s_R[f]	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)
P-value	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Candidate confirmed positive/total samples analyzed	0/144	143/144	144/144	0/144	143/144	144/144	0/144	143/144	144/144
Candidate confirmed POD (CC)	0.00 (0.00, +0.03)	0.99 (+0.96, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.99 (+0.96, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.99 (+0.96, +1.00)	1.00 (+0.97, +1.00)
s_r	0.00 (0.00, +0.16)	0.08 (+0.07, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.08 (+0.07, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.08 (+0.07, +0.16)	0.00 (0.00, +0.16)
s_L	0.00 (0.00, +0.16)	0.00 (0.00, +0.03)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.03)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.03)	0.00 (0.00, +0.16)
s_R	0.00 (0.00, +0.22)	0.08 (+0.08, +0.10)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.08 (+0.08, +0.10)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.08 (+0.08, +0.10)	0.00 (0.00, +0.22)
P-value	1.0000	0.4368	1.0000	1.0000	0.4368	1.0000	1.0000	0.4368	1.0000
Positive reference samples/total samples analyzed	0/144	84/144	138/144	0/144	84/144	138/144	0/144	84/144	138/144
Reference POD	0.00 (0.00, +0.03)	0.58 (+0.50, +0.67)	0.96 (+0.91, +0.98)	0.00 (0.00, +0.03)	0.58 (+0.50, +0.67)	0.96 (+0.91, +0.98)	0.00 (0.00, +0.03)	0.58 (+0.50, +0.67)	0.96 (+0.91, +0.98)
s_r	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.19 (+0.17, +0.22)	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.19 (+0.17, +0.22)	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.19 (+0.17, +0.22)
s_L	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.06 (+0.02, +0.13)	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.06 (+0.02, +0.13)	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.06 (+0.02, +0.13)
s_R	0.00 (0.00, +0.22)	0.50 (+0.45, +0.52)	0.20 (+0.18, +0.24)	0.00 (0.00, +0.22)	0.50 (+0.45, +0.52)	0.20 (+0.18, +0.24)	0.00 (0.00, +0.22)	0.50 (+0.45, +0.52)	0.20 (+0.18, +0.24)
P-value	1.0000	0.6298	0.0179	1.0000	0.6298	0.0179	1.0000	0.6298	0.0179
dLPOD (candidate vs reference)	0.00 (−0.03, +0.03)	0.41 (+0.32, +0.49)	0.04 (+0.01, +0.09)	0.00 (−0.03, +0.03)	0.41 (+0.32, +0.49)	0.04 (+0.01, +0.09)	0.00 (−0.03, +0.03)	0.41 (+0.32, +0.49)	0.04 (+0.01, +0.09)
dLPOD (candidate presumptive vs candidate confirmed)	0.00 (−0.03, +0.03)	0.01 (−0.02, +0.04)	0.00 (−0.03, +0.03)	0.00 (−0.03, +0.03)	0.01 (−0.02, +0.04)	0.00 (−0.03, +0.03)	0.00 (−0.03, +0.03)	0.01 (−0.02, +0.04)	0.00 (−0.03, +0.03)

[a] Results include 95% confidence intervals.

[b] Traditional confirmation on ASAP/IBISA = secondary enrichments streaked onto IBISA and ASAP.

[c] Alternative confirmation on ASAP/IBISA = direct streak of the primary enrichment onto IBISA and ASAP.

[d] Repeatability standard deviation.

[e] Among-laboratory standard deviation.

[f] Reproducibility standard deviation.

Table 2013.01B. Summary of results for the detection of *Salmonella* spp. in raw ground beef (375 g)

Method[a]	VIDAS SPT with traditional confirmation on BGSA and XLT4			VIDAS SPT with traditional confirmation on IBISA and ASAP[b]			VIDAS SPT with alternative confirmation on IBISA and ASAP[c]		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total samples analyzed	0/132	58/131	130/132	0/132	58/131	130/132	0/132	57/131	130/132
Candidate presumptive POD (CP)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.44 (+0.33, +0.54)	0.98 (+0.965, +1.00)
s_r[d]	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.44. +0.52)	0.12 (+0.11, +0.16)
s_L[e]	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.09 (0.00, +0.26)	0.00 (0.00, +0.05)
s_R[f]	0.00 (0.00, +0.23)	0.50 (+0.44, +0.52)	0.12 (+0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.44, +0.52)	0.12 (+0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (+0.11, +0.14)
P-value	1.0000	0.1551	0.5190	1.0000	0.1551	0.5190	1.0000	0.1906	0.5190
Candidate confirmed positive/total samples analyzed	0/132	58/131	130/132	0/132	59/131	130/132	0/132	58/131	130/132
Candidate confirmed POD (CC)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.45 (+0.35, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)
s_r	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.44, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)
s_L	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.09 (0.00, +0.25)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)
s_R	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (+0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (+0.11, +0.14)
P-value	1.0000	0.1551	0.5190	1.0000	0.2060	0.5190	1.0000	0.1551	0.5190
Positive reference samples/total samples analyzed	0/132	57/132	132/132	0/132	57/132	132/132	0/132	54/132	131/132
Reference POD	0.00 (0.00, +0.03)	0.43 (+0.35, +0.52)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.43 (+0.35, +0.52)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.41 (+0.32, +0.50)	0.99 (+0.96, +1.00)
s_r	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.49 (+0.44, +0.52)	0.09 (+0.08, +0.16)
s_L	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.05 (0.00, +0.22)	0.00 (0.00, +0.04)
s_R	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.23)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.23)	0.00 (0.00, +0.23)	0.49 (+0.44, +0.52)	0.09 (+0.08, +0.10)
P-value	1.0000	0.6261	1.0000	1.0000	0.6261	1.0000	1.0000	0.3313	0.4338
dLPOD (C vs R)	0.00 (−0.03, +0.03)	0.01 (−0.12, +0.15)	−0.02 (−0.05, +0.02)	0.00 (−0.03, +0.03)	0.02 (−0.18, +0.22)	−0.02 (−0.05, +0.02)	0.00 (−0.03, +0.03)	0.03 (−0.18, +0.24)	−0.01 (−0.05, +0.03)
dLPOD (CP vs CC)	0.00 (−0.03, +0.03)	0.00 (−0.15, +0.15)	0.00 (−0.04, +0.04)	0.00 (−0.03, +0.03)	−0.01 (−0.15, +0.14)	0.00 (−0.04, +0.04)	0.00 (−0.03, +0.03)	−0.01 (−0.21, +0.23)	0.00 (−0.04, +0.04)

[a] Results include 95% confidence intervals.

[b] Traditional confirmation on ASAP/IBISA = secondary enrichments streaked onto IBISA and ASAP.

[c] Alternative confirma ion = direct streak of the primary enrichment onto IBISA and ASAP.

[d] Repeatability standard deviation.

[e] Among-laboratory standard deviation.

[f] Reproducibility standard deviation.

Table 2013.01C.   Reagents included in 10-well reagent strip

Wells	Reagents (SPT)
1	Sample well: 0.5 mL of enrichment broth, standard or control
2	Prewash solution (400 µL): Buffer pH 7.8 + preservative
3–5, 7–9	Wash buffer (600 µL): TRIS-buffered saline (150 mmol/L) – Tween pH 7.6 + preservative
6	Conjugate (400 µL): alkaline phosphatase-labeled proteins specific for *Salmonella* receptors + preservative
10	Reading cuvette with substrate (300 µL): 4-methyl-umbelliferyl phosphate (0.6 mmol/L) + diethanolamine[a] (DEA; 0.62 mol/L or 6.6%, pH 9.2) + preservative

[a]   Irritant reagent; *see* VIDAS SPT package insert for more information.

Manual). Remove the strip and allow to cool for 10 min prior to test initiation. Perform the VIDAS test.

E. Enzyme Immunoassay

(**a**) Enter factory master calibration curve data into the instrument using the MLE card.

(**b**) Remove the kit reagents and materials from refrigerated storage and allow them to come to room temperature.

(**c**) Use one VIDAS SPT reagent strip and one VIDAS SPT SPR for each sample, control, or standard to be tested. Reseal the storage pouch after removing the required number of SPRs.

(**d**) Enter the appropriate assay information to create a work list. Enter the test code by typing or selecting "SPT," and number of tests to be run. If the standard is to be tested, identify the standard by "S1" and test in duplicate. If the positive control is to be tested, identify it by "C1." If the negative control is to be tested, identify it by "C2."

Note: The standard must be tested upon receipt of a new lot of reagents and then every 14 days. The relative fluorescence value (RFV) of the standard must fall within the set range provided with the kit.

(**e**) Load the SPT reagents strips and SPRs into the positions that correspond to the VIDAS section indicated by the work list. Verify that the color labels with the assay code on the SPRs and reagent strips match.

(**f**) Initiate the assay processing as directed in the VIDAS operator's manual.

(**g**) After the assay is completed, remove the SPRs and reagent strips from the instrument and dispose of properly.

Table 2013.01D.   Interpretation of test

Test value threshold	Interpretation
<0.25	Negative
≥0.25	Positive

F. Results and Interpretation

The results are analyzed automatically by the VIDAS system. A report is printed which records the type of test performed, test sample identification, date and time, lot number, and expiration date of the reagent kit being used, each sample's RFV, test value, and interpreted result (positive or negative). Fluorescence is measured twice in the reagent strip's reading cuvette for each sample tested. The first reading is a background reading of the substrate cuvette before the SPR is introduced into the substrate. The second reading is taken after incubating the substrate with the enzyme remaining on the interior of the SPR. The test value is calculated by the instrument and is equal to the difference between the background reading and the final reading. The calculation appears on the result sheet. A negative result has a test value less than the threshold (0.25) and indicates that the sample does not contain *Salmonella* spp. or contains *Salmonella* spp. at a concentration below the detection limit. A positive result has a test value equal to or greater than the threshold (≥0.25) and indicates that the sample may be contaminated with *Salmonella* spp. If the background reading is above a predetermined cutoff, then the result is reported as invalid (Table **2012.01D**).

G. Confirmation

All positive VIDAS SPT results must be culturally confirmed. Confirmation should be performed using the non-heated enrichment broth stored between 2 and 8°C, and should be initiated within 72 h after the end of incubation at 42 ± 1°C. Presumptive positive results may be confirmed by isolating on selective agar plates such as IBISA or ASAP, or on the appropriate reference method selective agar plates. Typical or suspect colonies from each plate are confirmed as described in **967.27** (*see* 17.9.03). As an alternative to the conventional tube system for *Salmonella*, any AOAC-approved commercial biochemical kits may be used for presumptive generic identification of foodborne *Salmonella* as described in **978.24** (*see* 17.9.04), **989.12** (*see* 17.9.05), **991.13** (*see* 17.9.06), and **2011.17** (*see* 17.15.01).

Reference: *J. AOAC Int*. **96**, 808(2013)
          DOI: 10.5740/jaoacint.CS2013_01

BAM: Clostridium botulinum

January 2001

Bacteriological Analytical Manual
Chapter 17
Clostridium botulinum

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Clostridium botulinum is an anaerobic, rod-shaped sporeforming bacterium that produces a protein with characteristic neurotoxicity. Under certain conditions, these organisms may grow in foods producing toxin(s). Botulism, a severe form of food poisoning results when the toxin-containing foods are ingested. Although this food illness is rare, its mortality rate is high; the 962 recorded botulism outbreaks in the United States from 1899 to 1990 (2) involved 2320 cases and 1036 deaths. In outbreaks in which the toxin type was determined, 384 were caused by type A, 106 by type B, 105 by type E, and 3 by type F. In two outbreaks, the foods implicated contained both types A and B toxins. Due to a limited number of reports, type C and D toxins have been questioned as the causative agent of human botulism. It is suspected that these toxins are not readily absorbed in the human intestine. However, all types except F and G, which have not been as studied thoroughly, are important causes of animal botulism.

Antigenic types of *C. botulinum* are identified by the complete neutralization of their toxins using the homologous antitoxin. Cross-neutralization of a specific toxin by heterologous antitoxins does not occur or is minimal. There are seven recognized antigenic types: A through G. Cultures of five of these types apparently produce only one type of toxin but all are given type designations corresponding to their toxin production. Types C and D cross-react with antitoxins to each other because they each produce more than one toxin and have at least one common toxin component. Type C produces predominantly C_1 toxin with lesser amounts of D and C_2, or only C_2, and type D produces predominantly type D toxin along with smaller amounts of C_1 and C_2. Mixed toxin production by a single strain of *C. botulinum* may be more common than previously realized. There is a slight reciprocal cross-neutralization with types E and F, and recently a strain of *C. botulinum* was shown to produce a mixture of predominantly type A toxin, with a small amount of type F.

Aside from toxin type, *C. botulinum* can be differentiated into general groups on the basis of cultural, biochemical, and physiological characteristics. Cultures producing types C and D toxins are not proteolytic on coagulated egg white or meat and have a common metabolic pattern which sets them apart from the others. All cultures that produce type A toxin and some that produce B and F toxins are proteolytic. All type E strains and the remaining B and F strains are nonproteolytic, with carbohydrate metabolic patterns differing from the C and D nonproteolytic groups. Strains that produce type G toxin have not been studied in sufficient detail for effective and satisfactory characterization.

C. botulinum is widely distributed in soils and in sediments of oceans and lakes. The finding of type E in aquatic environments by many investigators correlates with cases of type E botulism that were traced to

contaminated fish or other seafoods. Types A and B are most commonly encountered in foods associated with soil contamination. In the United States, home-canned vegetables are most commonly contaminated with types A and B, but in Europe, meat products have also been important vehicles of foodborne illness caused by these types.

Measures to prevent botulism include reduction of the microbial contamination level, acidification, reduction of moisture level, and whenever possible, destruction of all botulinal spores in the food. Heat processing is the most common method of destruction. Properly processed canned foods will not contain viable *C. botulinum*. Home-canned foods are more often a source of botulism than are commercially canned foods, which probably reflects the commercial canners' great awareness and better control of the required heat treatment.

A food may contain viable *C. botulinum* and still not be capable of causing botulism. If the organisms do not grow, no toxin is produced. Although many foods satisfy the nutritional requirements for the growth of *C. botulinum*, not all of them provide the necessary anaerobic conditions. Both nutritional and anaerobic requirements are supplied by many canned foods and by various meat and fish products. Growth in otherwise suitable foods can be prevented if the product, naturally or by design, is acidic (of low pH), has low water activity, a high concentration of NaCl, an inhibitory concentration of $NaNO_2$ or other preservative, or two or more of these conditions in combination. Refrigeration will not prevent growth and toxin formation by nonproteolytic strains unless the temperature is precisely controlled and kept below 3°C. Foods processed to prevent spoilage but not usually refrigerated are the most common vehicles of botulism.

Optimum temperature for growth and toxin production of proteolytic strains is close to 35°C; for nonproteolytic strains it is 26-28°C. Nonproteolytic types B, E, and F can produce toxin at refrigeration temperatures (3-4°C). Toxins of the nonproteolytics do not manifest maximum potential toxicity until they are activated with trypsin; toxins of the proteolytics generally occur in fully (or close to fully) activated form. These and other differences can be important in epidemiological and laboratory considerations of botulism outbreaks. Clinical diagnosis of botulism is most effectively confirmed by identifying botulinal toxin in the blood, feces, or vomitus of the patient. Specimens must be collected before botulinal antitoxin is administered to the patient. Identifying the causative food is most important in preventing additional cases of botulism. See Examination of Canned Foods, Chapter 21.

Botulism in infants 6 weeks to 1 year of age was first recognized as a distinct clinical entity in 1976. This form of botulism results from growth and toxin production by *C. botulinum* within the intestinal tract of infants rather than from ingestion of a food with preformed toxin. It is usually caused by *C. botulinum* types A or B, but a few cases have been caused by other types. Infant botulism has been diagnosed in most U.S. states and in every populated continent except Africa (1).

Constipation almost always occurs in infant botulism and usually precedes characteristic signs of neuromuscular paralysis by a few days or weeks. Illnesses have a broad range of severity. Some infants show only mild weakness, lethargy, and reduced feeding and do not require hospitalization. Many have shown more severe symptoms such as weakened suck, swallowing, and cry; generalized muscle weakness; and diminished gag reflex with a pooling of oral secretions. Generalized muscle weakness and loss of head control in some infants reaches such a degree of severity that the patient appears "floppy." In some hospitalized cases, respiratory arrest has occurred, but most were successfully

resuscitated, and with intense supportive care have ultimately recovered. As a result, the case-fatality rate (2%) for this form of botulism is low. Recovery usually requires at least several weeks of hospitalization (1).

Honey, a known source of *C. botulinum* spores, has been implicated in some cases of infant botulism. In studies of honey, up to 13% of the test samples contained low numbers of *C. botulinum* spores (3). For this reason, the FDA, the Centers for Disease Control and Prevention (CDC), and the American Academy of Pediatrics recommend not feeding honey to infants under one year old.

The mouse bioassay is a functional assay that detects biologically active toxin. The assay requires a three part approach: toxin screening, toxin titer, and finally toxin neutralization using monovalent antitoxins. The process requires two days of analysis at each step.

Recently, rapid, alternative, in-vitro procedures have been developed for the detection of types A, B, E, and F botulinal toxin producing organisms and their toxins. The toxins generated in culture media can be detected using ELISA techniques such as the DIG-ELISA and the amp-ELISA. Biologically active and non-active toxins are detected since the assay detects the toxin antigen. The ELISA assays require one day of analysis. The toxin genes of viable organisms can be detected using the polymerase chain reaction technique and require one days of analysis after overnight incubation of botulinal spores or vegetative cells. In-vitro assays that are positive are confirmed using the mouse bioassay.

I. **Mouse Bioassay for *Clostridium botulinum* Toxin**

    A. Equipment and Materials

        1. Refrigerator

        2. Clean dry towels

3. Bunsen burner

4. Sterile can opener (bacteriological or puncture type)

5. Sterile mortar and pestle

6. Sterile forceps

7. Sterile cotton-plugged pipets

8. Mechanical pipetting device (**NEVER** pipet by mouth)

9. Sterile culture tubes (at least a few should be screw-cap tubes)

10. Anaerobic jars (GasPak or Case-nitrogen replacement)

11. Transfer loops

12. Incubators, 35 and 28°C

13. Sterile, reserve sample jars

14. Culture tube racks

15. Microscope slides

16. Microscope, phase-contrast or bright-field

17. Sterile petri dishes, 100 mm

18. Centrifuge tubes

19. Centrifuge, refrigerated, high-speed

20. Trypsin (1:250; Difco Laboratories, Detroit, MI)

21. Syringes, 1 and or 3 ml, sterile, with 25 gauge, 5/8 inch needles for injecting mice

22. Mice, 16-24 g (for routine work, up to 34 g)

23. Mouse cages, feed, water bottles, etc.

24. Millipore filters: 0.45 μm pore size

B. Media (/food/laboratory-methods/media-index-bam) and Reagents (/food/laboratory-methods/reagents-index-bam)

1. Alcoholic solution of iodine (4% iodine in 70% ethanol) (R18 (/food/laboratory-methods/bam-r18-disinfectants))

2. Chopped liver broth (M38 (/food/laboratory-methods/bam-media-m38-chopped-liver-broth)) or cooked meat medium (M42 (/food/laboratory-methods/bam-media-m42-cooked-meat-medium))

3. Trypticase-peptone-glucose-yeast extract (TPGY) (M151 (/food/laboratory-methods/bam-media-m151-trypticase-peptone-glucose-yeast-extract-broth-tpgy)) broth or with trypsin (TPGYT) (M151a (/food/laboratory-methods/bam-media-m151a-trypticase-peptone-glucose-yeast-extract-broth-trypsin-tpgyt))

4. Liver-veal-egg yolk agar (M84 (/food/laboratory-methods/bam-media-m84-liver-veal-egg-yolk-agar)) or anaerobic egg yolk agar (M12 (/food/laboratory-methods/bam-media-m12-anaerobic-egg-yolk-agar))

5. Sterile, gel-phosphate buffer, pH 6.2 (R29 (/food/laboratory-methods/bam-r29-gel-phosphate-buffer))

6. Absolute ethanol

7. Gram stain reagents (R32 (/food/laboratory-methods/bam-r32-gram-stain)), crystal violet (R16 (/food/laboratory-methods/bam-r16-crystal-violet-stain-bacteria)), or methylene blue (R45 (/food/laboratory-methods/bam-r45-methylene-blue-stain-loefflers)) solutions

8. Sterile physiological saline solution (R63 (/food/laboratory-methods/bam-r63-physiological-saline-solution-085-sterile))

9. Monovalent antitoxin preparations, types A-F (obtain from CDC)

10. Trypsin solution (prepared from Difco 1:250)

11. 1 N Sodium hydroxide solution (R73 (/food/laboratory-methods/bam-r73-1-n-sodium-hydroxide-solution))

12. 1 N Hydrochloric acid solution (R36 (/food/laboratory-methods/bam-r36-1-n-hydrochloric-acid))

C. Sample preparation

Preliminary examination. Refrigerate samples until testing, except unopened canned foods, which need not be refrigerated unless badly swollen and in danger of bursting. Before testing, record product designation, manufacturer's name or home canner, source of sample, type of container and size, labeling, manufacturer's batch, lot or production code, and condition of container. Clean and mark container with laboratory identification codes.

Solid and liquid foods. Aseptically transfer foods with little or no free liquid to sterile mortar. Add equal amount of gel-phosphate buffer solution and grind with sterile pestle before inoculation. Alternatively, inoculate small pieces of product directly into enrichment broth with sterile forceps. Inoculate liquid foods directly into enrichment broth with sterile pipets. Reserve sample; after culturing, aseptically remove reserve portion to sterile sample jar for tests which may be needed later. Refrigerate reserve sample.

Opening of canned foods (**see** Chapter 21).

Examine product for appearance and odor. Note any evidence of decomposition. **DO NOT TASTE** the product under any circumstances. Record the findings.

D. Detection of viable *C. botulinum*

1. **Enrichment**. Remove dissolved oxygen from enrichment media by steaming 10-15 min and cooling quickly without agitation before inoculation.

   Inoculate 2 tubes of cooked meat medium with 1-2 g solid or 1-2 ml liquid food per 15 ml enrichment broth. Incubate at 35°C.

   Inoculate 2 tubes of TPGY broth as above. Incubate at 28°C. Use TPGYT as alternative only when organism involved is strongly suspected of being a nonproteolytic strain of types B, E, or F.

   Introduce inoculum slowly beneath surface of broth to bottom of tube. After 5 days of incubation, examine enrichment cultures. Check for turbidity, gas production, and digestion of meat particles. Note the odor.

   Examine cultures microscopically by wet mount under high-power phase contrast, or a smear stained by Gram reagent, crystal violet, or methylene blue under bright-field illumination. Observe morphology of organisms and note existence of typical clostridial cells, occurrence and relative extent of sporulation, and location of spores within cells. A typical clostridial cell resembles a tennis racket. At this time test each enrichment culture for toxin, and if present, determine toxin type according to procedure in F, below. Usually, a 5-day incubation is the period of active growth giving the highest concentration

of botulinal toxin. If enrichment culture shows no growth at 5 days, incubate an additional 10 days to detect possible delayed germination of injured spores before discarding sample as sterile. For pure culture isolation save enrichment culture at peak sporulation and keep under refrigeration.

2. **Isolation of pure cultures.** *C. botulinum* is more readily isolated from the mixed flora of an enrichment culture or original specimen if sporulation has been good.

   Pre-treatment of specimens for streaking. Add equal volume of filter-sterilized absolute alcohol to 1 or 2 ml of enrichment culture in sterile screw-cap tube. Mix well and incubate 1 h at room temperature. To isolate from sample, take 1 or 2 ml of retained portion, and add an equal volume of filter-sterilized absolute alcohol in sterile screw-cap tube. Mix well and incubate 1 h at room temperature. Alternatively, heat 1 or 2 ml of enrichment culture or sample to destroy vegetative cells (80°C for 10-15 min). **DO NOT** use heat treatment for nonproteolytic types of *C. botulinum.*

   Plating of treated cultures. With inoculating loop, streak 1 or 2 loopfuls of ethanol or heat-treated cultures to either liver- veal-egg yolk agar or anaerobic egg yolk agar (or both) to obtain isolated colonies. If necessary, dilute culture to obtain well-separated colonies. Dry agar plates well before use to prevent spreading of colonies. Incubate streaked plates at 35°C for about 48 h under

anaerobic conditions. A Case anaerobic jar or the GasPak system is adequate to obtain anaerobiosis; however, other systems may be used.

E. Selection of typical *C. botulinum* colonies

Selection. Select about 10 well-separated typical colonies, which may be raised or flat, smooth or rough. Colonies commonly show some spreading and have an irregular edge. On egg yolk medium, they usually exhibit surface iridescence when examined by oblique light. This luster zone, often referred to as a pearly layer, usually extends beyond and follows the irregular contour of the colony. Besides the pearly zone, colonies of *C. botulinum* types C, D, and E are ordinarily surrounded by a wide zone (2-4 mm) of yellow precipitate. Colonies of types A and B generally show a smaller zone of precipitation. Considerable difficulty may be experienced in picking toxic colonies since certain other members of the genus *Clostridium* produce colonies with similar morphological characteristics but do not produce toxins.

Inoculation. Use sterile transfer loop to inoculate each selected colony into tube of sterile broth. Inoculate *C. botulinum* type E into TPGY broth. Inoculate other toxin types of *C. botulinum* into chopped liver broth or cooked meat medium. Incubate as described in D-1, above, for 5 days. Test for toxin production as described in F, below. To determine toxin type, **see** F-3, below.

Isolation of pure culture. Restreak toxic culture in duplicate on egg yolk agar medium. Incubate one plate anaerobically at 35°C. Incubate second plate aerobically at 35°C. If colonies typical of *C. botulinum* are found only on anaerobic plate (no growth on aerobic plate), the culture may

be pure. Failure to isolate *C. botulinum* from at least one of the selected colonies means that its population in relation to the mixed flora is probably low. Repeated serial transfer through additional enrichment steps may increase the numbers sufficiently to permit isolation. Store pure culture in sporulated state either under refrigeration, on glass beads, or lyophilized.

F. Detection and identification of botulinal toxin

1. **Preparation of food sample**. Culture one portion of sample for detection of viable *C. botulinum*; remove another portion for toxicity testing, and store remainder in refrigerator. Centrifuge samples containing suspended solids under refrigeration and use supernatant fluid for toxin assay. Extract solid foods with equal volume of gel-phosphate buffer, pH 6.2, by macerating food and buffer with pre-chilled mortar and pestle. Centrifuge macerated sample under refrigeration and use supernatant fluid for toxin assay. Rinse empty containers suspected of having held toxic foods with a few milliliters of gel-phosphate buffer. Use as little buffer as possible to avoid diluting toxin beyond detection. To avoid or minimize nonspecific death of mice, filter supernatant fluid through a millipore filter before injecting mice. For non-proteolytic samples or cultures, trypsinize after filtration.

2. **Determination of toxicity in food samples or cultures**

   Trypsin treatment. Toxins of nonproteolytic types, if present, may need trypsin activation to be detected. Therefore, treat a portion of food supernatant fluid, liquid food, or TPGY culture with trypsin before testing

for toxin. Do not treat TPGYT culture with trypsin since this medium already contains trypsin and further treatment may degrade any fully activated toxin that is present. Adjust portion of supernatant fluid, if necessary, to pH 6.2 with 1 N NaOH or HCl. Add 0.2 ml aqueous trypsin solution to 1.8 ml of each supernatant fluid to be tested for toxicity. (To prepare trypsin solution, place 0.5 g of Difco 1:250 trypsin in clean culture tube and add 10 ml distilled water, shake, and warm to dissolve. Analysts who are allergic to trypsin should weigh it in a hood or wear a face mask.) Incubate trypsin- treated preparation at 35-37°C for 1 h with occasional gentle agitation.

Toxicity testing. Conduct parallel tests with trypsin-treated materials and untreated duplicates. Dilute a portion of untreated sample fluid or culture to 1:5, 1:10, and 1:100 in gel-phosphate buffer. Make the same dilutions of each trypsinized sample fluid or culture. Inject each of separate pairs of mice intraperitoneally (i.p.) with 0.5 ml untreated undiluted fluid and 0.5 ml of each dilution of untreated test sample, using a 1 or 3 ml syringe with 5/8 inch, 25 gauge needle. Repeat this procedure with trypsin-treated duplicate samples. Heat 1.5 ml of untreated supernatant fluid or culture for 10 min at 100°C. Cool heated sample and inject each of a pair of mice with 0.5 ml undiluted fluid. These mice should not die, because botulinal toxin, if present, will be inactivated by heating.

Observe all mice periodically for 48 h for symptoms of botulism. Record symptoms and deaths. Typical botulism signs in mice begin usually in the first 24 h with ruffling of fur, followed in sequence by labored

breathing, weakness of limbs, and finally total paralysis with gasping for breath, followed by death due to respiratory failure. Death of mice without clinical symptoms of botulism is not sufficient evidence that injected material contained botulinal toxin. On occasion, death occurs from other chemicals present in injected fluid, or from trauma.

If after 48 h of observation, all mice except those receiving the heated preparation have died, repeat the toxicity test, using higher dilutions of supernatant fluids or cultures. It is necessary to have dilutions that kill and dilutions that do not kill in order to establish an endpoint or the minimum lethal dose (MLD) as an estimate of the amount of toxin present. The MLD is contained in the highest dilution killing both mice (or all mice inoculated). From these data, the number of MLD/ml can be calculated.

3. **Typing of toxin**. Rehydrate antitoxins with sterile physiological saline. **Do not use glycerin water**. Dilute monovalent antitoxins to types A, B, E, and F in physiological saline to contain 1 international unit (IU) per 0.5 ml. Prepare enough of these antitoxin solutions to inject 0.5 ml of antitoxin into each of 2 mice for each dilution of toxic preparation to be tested. Use the toxic preparation that gave the higher MLD, either untreated or trypsinized. Prepare dilutions of the toxic sample to cover at least 10, 100, and 1000 MLD below the previously determined endpoint of toxicity if possible (see 2, above). The untreated toxic preparation can be the same as that used for testing toxicity. If a trypsinized

preparation was the most lethal, it will be necessary to prepare a freshly trypsinized fluid. The continued action of trypsin may destroy the toxin.

Inject the mice with the monovalent antitoxins, as described above, 30 min to 1 h before challenging them with i.p. injection of the toxic preparations. Inject pairs of mice (protected by specific monovalent antitoxin injection) i.p. with each dilution of the toxic preparation. Also inject a pair of unprotected mice (no injection of antitoxin) with each toxic dilution as a control. The use of 4 monovalent antitoxins (types A, B, E, and F) for the unknown toxic sample prepared at 3 dilutions requires a total of 30 mice — 6 mice for each antitoxin (24 mice) plus 2 unprotected mice for each of the 3 dilutions (6 mice) as controls. Observe mice for 48 h for symptoms of botulism and record deaths. If test results indicate that toxin was not neutralized, repeat test, using monovalent antitoxins to types C and D, plus polyvalent antitoxin pool of types A through F.

II. Mouse Screening Procedure for *Clostridium botulinum* Type E Spores in Smoked Fish

A. Equipment and Materials

1. 12 mice (16-24 g, or up to 34 g) per subsample (24 or more required for positives)

2. Types A, B, E antisera

3. Saline, sterile, 0.85% NaCl (R63 (/food/laboratory-methods/bam-r63-physiological-saline-solution-085-sterile))

4. Trypsin (Difco); 1:250, 5% solution

5. Syringes, 1 and 3 ml, 25 gauge, 5/8 inch needle

6. Incubator 28°C

7. TPGY medium (M151 (/food/laboratory-methods/bam-media-m151-trypticase-peptone-glucose-yeast-extract-broth-tpgy))

8. Water bath, 37°C

9. Gel-phosphate diluent (R29 (/food/laboratory-methods/bam-r29-gel-phosphate-buffer))

10. Centrifuge, refrigerated

11. Plastic bags, strong and water-tight

B. Procedure

Incubation. Place each smoked fish subsample (which may consist of 1 or more fish, depending on size, and may be either vacuum-packed or bulk-smoked fish) in a strong water-tight plastic bag. Add freshly steamed and cooled TPGY broth to subsample. **NOTE**: Add enough TPGY broth to completely cover fish. Squeeze bag to expel as much air as possible and seal it with hot-iron bag sealer or other air-tight closure device. Incubate at 28°C for 5 days. Precautions should be taken during incubation period since bag may swell and split from gas formation.

Cultures. At end of incubation period, centrifuge 20 ml of TPGY culture from each subsample at $7500 \times g$ rpm for 20 min. Use refrigerated centrifuge. Determine pH of TPGY. If above 6.5, adjust to 6.0-6.2 with HCl. Refrigerate for overnight storage.

Trypsinization. To 3.6 ml of culture, adjusted to pH 6.0-6.2, add 0.4 ml of 5% solution of trypsin. Incubate at 35-37°C for 1 h. Remove culture and let cool to room temperature before injecting mice. Trypsinized extract cannot be stored overnight.

Toxicity screening. Dilute trypsinized and nontrypsinized broth cultures to 1:5, 1:10, and 1:100 in gel-phosphate diluent. (**NOTE**: Do not store trypsinized material overnight.) Inject mice i.p. with 0.5 ml of each dilution. Inject 2 mice per dilution, i.e., trypsinized and nontrypsinized (total 12 mice per subsample). Observe mice for botulism symptoms and record condition of mice at frequent intervals for 48 h. If no deaths occur, no further tests are indicated. Deaths are presumptive evidence of toxin and should be confirmed.

Confirmation with protected mice. Dilute new portion of nontrypsinized or trypsinized culture (whichever showed the highest titer) to 1:5, 1:10, and 1:100 in gel-phosphate diluent. (Do not store trypsinized material overnight.) Inject 6 mice i.p. with 0.5 ml of 1:5 saline dilution of type E antiserum. These will be compared to 6 mice without this protection (controls). After 30 min, inject 0.5 ml of each dilution into 2 mice protected with antiserum and into 2 mice not so protected. Record their condition at intervals up to 48 h. If unprotected mice die and protected mice live, the presence of type E toxin is indicated. If all protected mice die, repeat confirmation with higher dilutions of toxic culture in type E-protected mice and with mice protected against *C. botulinum* types A and/or B antiserum. If all antiserum-protected mice die, send toxic culture media on dry ice to Division of Microbiological Studies (HFS-516), FDA, *5100 Paint Branch Pkwy, College Park, MD 20740*, for further tests. Isolate and identify cultures from samples containing toxin of type E, if possible.

Obtain *C. botulinum* antisera from Centers for Disease Control and Prevention, Atlanta, GA 30333, USA. Reconstitute lyophilized antisera with sterile saline. Dilute sera 1:5 with sterile saline for mouse injection.

If you have questions about the method, contact **Shashi Sharma**, FDA. Telephone **(240)-402-1570**.

General Hints Regarding *C. botulinum* Toxin Analysis

1. The first 24 hours are the most important time regarding symptoms and death of mice: 98-99% of animals die within 24 hours. Typical symptoms of botulism and death may occur within 4 to 6 hours.

2. If deaths occur after 24 hours, be very suspicious, unless typical botulism symptoms are clearly evident.

3. If deaths occur in mice injected with the 1:2 or 1:5 dilution but not with any higher dilution, be very suspicious. Deaths may have been from nonspecific causes.

4. Mice can be marked on tails with dye to represent various dilutions. Dye does not come off easily.

5. Mice injected with botulinal toxin may become hyperactive before symptoms occur.

6. Food and water may be given to the mice right away; it will not interfere with the test.

7. Rehydrated antitoxin may be kept up to 6 months under refrigeration, and may be frozen indefinitely.

8. TPGY medium is relatively stable and can be kept 2-3 weeks under refrigeration.

9. With cooked meat medium, vortex tubes completely; toxin may adhere to meat particles.

10. Trypsin is not filtered. Use 0.5 g in 10 ml of distilled water. It can be kept up to 1 week under refrigeration.

Interpretation of Data (NOTE: Laboratory tests are designed to identify botulinal toxin and/or organisms in foods)

1. Toxin in a food means that the product, if consumed without thorough heating, could cause botulism.

2. Viable *C. botulinum* but no toxin in foods is not proof that the food in question caused botulism.

3. The presence of toxin in food is required for an outbreak of botulism to occur.

4. Ingested organisms may be found in the alimentary tract, but are considered to be unable to multiply and produce toxin in vivo, except in infants.

5. Presence of botulinal toxin and/or organisms in low-acid (i.e., above pH 4.6) canned foods means that the items were underprocessed or were contaminated through post-processing leakage.

   - Swollen cans are more likely than flat cans to contain botulinal toxin since the organism produces gas during growth.

   - Presence of toxin in a flat can may imply that the seams were loose enough to allow gas to escape.

   - Botulinal toxin in canned foods is usually of a type A or a proteolytic type B strain, since spores of the proteolytics can be among the more heat-resistant.

   - Spores of nonproteolytics, types B, E, and F, generally are of low heat resistance and would not normally survive even mild heat treatment.

6. The protection of mice from botulism and death with one of the

monovalent botulinal antitoxins confirms the presence of botulinal toxin and determines the serological type of toxin in a sample.

7. The following reasons may explain why deaths occur in mice that are protected by one of the monovalent antitoxins:

    - There may be too much toxin in the sample.

    - More than one kind of toxin may be present.

    - Deaths may be due to some other cause.

    Retesting at higher dilutions of toxic fluids is required, and mixtures of antitoxins must be used in place of monovalent antiserum. Some other toxic material, which is not heat-labile, could be responsible if both heated and unheated fluids cause death. The heat-stable toxic substance could possibly mask botulinal toxin.

Safety Precautions for the *Clostridium botulinum* Laboratory

1. Place biohazard signs on doors to restrict entrance and keep the number of people in the laboratory to a minimum.

2. All workers in the laboratory should wear laboratory coats and safety glasses.

3. Use 1% hypochlorite solution to wipe laboratory table tops before and after work.

4. **NEVER PIPETTE ANYTHING BY MOUTH. USE MECHANICAL PIPETTORS.**

5. Use a biohazard hood for transfer of toxic material, if possible.

6. Centrifuge toxic materials in a hermetically closed centrifuge with safety cups.

7. Personally take all toxic material to the autoclave and see that it is sterilized immediately.

8. Do not work alone in the laboratory or animal rooms after hours or on weekends.

9. Have an eye wash fountain and foot-pedaled faucet available for hand washing.

10. No eating and drinking in the laboratory when someone works with toxins.

11. In a very visible location, list phone numbers where therapeutic antitoxin can be obtained in case of emergency. **THIS IS VERY IMPORTANT!**

12. Reduce clutter in the laboratory to a minimum and place equipment and other materials in their proper place after use.

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3. Hauschild, A.H.W., R. Hilsheimer, K.F. Weiss, and R.B. Burke. 1988. *Clostridium botulinum* in honey, syrups, and dry infant cereals. *J. Food Prot.* **51**:892-894.

III. **Amplified ELISA Procedure for Detection of Botulinal Toxins A, B, E, and F from Culture.** Contact Joseph L. Ferreira (404 253-2216) for questions about method.

These toxins can be detected using an amplified ELISA procedure that has a detection limit of approximately 10 MLD/mL. Toxic cultures may be more antigenic than purified toxins and the level of detection using the ELISA may be more sensitive than the mouse bioassay. Both TPGY and CMM are tested since more toxin may be generated in one medium compared to the other and the mouse bioassay, which is needed for confirmation of ELISA tests, also utilizes these media.

A. **Equipment and Materials**

1. Microplate, Dynex Immulon ll U-bottom, cat. No. 3655
2. Microtiter pipettors to deliver from 0.1- 2.0, 2-20, and 50-200 µl.
3. Multichannel pipettor, 8 or 12 place 50-200 µl
4. Pipets, disposable 1,5,10 ml
5. Glass test tubes 13X100 mm, 15X150 mm
6. Incubator, 35°C
7. Refrigerated centrifuge
8. Microplate washer
9. Microplate shaker
10. Microplate reader (read 490 and 630 nm reference)
11. Microtiter plate seals
12. Multichannel pipet reservoirs

B. Media (/food/laboratory-methods/media-index-bam) and Reagents (/food/laboratory-methods/reagents-index-bam)

1. Tryptone-peptone-glucose-yeast extract broth (TPGY).
2. Cooked meat medium (CMM).

3. 0.05M bicarbonate buffer: 0.8g Na_2CO_3 + 1.47g $NaHCO_3$ in 500 ml distilled H_2O, pH 9.6.

4. 1% Casein buffer: Add 10.0g vitamin-free casein + 7.65 g NaCl, 0.724g Na_2HPO_4 (anhydrous), 0.21g KH_2PO_4 to 900 ml H_2O, and 3 ml of 1 M NaOH. Heat with stirring to ~ 80°C to dissolve casein. Check pH and adjust to 7.9 with 1 M NaOH, q.s. to 1 liter. Sterilize at 121°C for 20 min. Final pH is ~7.4-7.6.

5. Goat type A or E, rabbit type B, or horse F antitoxin.

6. Goat type A, B, E, or F biotinylated antitoxin

7. Tris buffered NaCl-0.005% Tween 20 (TBST): 6.04g Tris base, 8.76g NaCl, Distilled H_2O 900 ml, dissolve Tris and NaCl, pH adjust to 7.5 at 25°C with 2 M HCl, add 50 μl of Tween-20 and q.s. to 1 liter.

8. Extravidin-alkaline phosphatase conjugate (Sigma)

9. Amplified ELISA substrate system (GibCo)

10. 0.3 M H_2SO_4: dilute concentrated acid (MW 98, specific gravity 1.84, purity 96-98%) by adding 1 ml to 59 ml of distilled H_2O.

11. Botulinal complex toxin standards A, B, E, and F. (Metabiologics Inc., Madison, WI)

C. Amplified ELISA Procedure

1. **Preparation of samples.** Food samples or anaerobic isolates picked from agar plates are inoculated into TPGY (**without trypsin**) and CMM as recommended in Chapter 17 of the Bacteriological Analytical Manual (2001). TPGY broth and cooked meat media are incubated for 5 days at 26°C and 35°C respectively.

Cultures are centrifuged at 7,000 × g and 4°C for 30 min, supernatant pH is adjusted to 7.4-7.6 using 1 N NaOH or 1N HCl. Samples and controls are analyzed **in duplicate** for TPGY and for CMM. Analyze undiluted and 1:5 dilutions of each culture supernatant. 1:5= 0.2 ml culture + 0.8 ml casein buffer.

2. **Preparation of microtiter plates.** Coat each well of the microtiter plate with 100 µl of appropriate dilution of goat type A, E, or F or rabbit type B antitoxin diluted in bicarbonate buffer. Prepare the number of needed microtiter plate wells to test the sample. Dilute the stock antitoxins according to the accompanying directions. Store plate with coating buffer overnight at 4°C with plastic seal cover on top of plate to prevent drying.

3. **ELISA analysis of culture media.**

   a. Remove plate from 4°C storage and wash plate 5 times in Tris buffered saline (TBST) with 45 second hold between each aspiration. Use a commercial plate washer or other mechanical device; avoid using a squeeze bottle to wash.

   b. Block plate in casein buffer with by filling all wells to the top of the plate (~300 µl/well) and incubate for 60-90 min at 35°C. Prepare the sample and control dilutions while the plate is being blocked.

   Negative controls: Duplicate wells with all reagents except toxin (undiluted sterile CMM and TPGY broth).

Positive controls: Test standard toxins type A, B, E, and F diluted in sterile TPGY and CMM (pH 7.6) at a concentration of 2 ng/ml (~2-60 LD_{50}/ng depending on toxin type).

c. Wash the blocked plate as above and then add the toxic samples and controls (100 μl/well). Work from the left side of the plate to the right side when adding the reagents.

d. Incubate toxin-containing samples and controls for 2 hr. at 35°C. Prepare the type A, B, E, and F biotin-labeled antibody reagents according to directions while incubating the samples. Do not make more than you need!

e. Wash plate 5 times in TBST as above.

f. Add the diluted biotin-labeled goat antibody (100 μl/well) and incubate for 60 min at 35°C.

g. Wash plate 5 times in TBST as above.

h. Add the streptavidin-alkaline phosphatase conjugate diluted 1:10,000 in casein buffer (100 μl/well), and incubate for 60 min at 35°C.

i. Wash 5 times in TBST with a final 10 minute soak (the last buffer wash is not aspirated). After 10 minute soak, discard the wash and tamp the plate several times on a paper towel to remove wash buffer.

j. Add 50 μl of the GIBCO substrate solution, incubate 12.5 min at room temperature on plate shaker (~100 rpm) then add 50 μl of the GIBCO amplifier and incubate for approximately an

additional 10 min. without shaking. The plate should be taken to the plate reader immediately after addition of the amplifier reagent and be ready to read the reactions. Read absorbance at 490 nm with 630 nm subtraction (reference filter) to account for plate absorbance. The analysis can be stopped at any time (2-15 min) after addition of the amplifier when positive controls give appropriate sensitivity (absorbance ≥ 1.0) and negative controls are acceptable (absorbance not greater than ~ 0.30). The reaction can be stopped with 50 μl of 0.3 M H_2SO_4 and the absorbance read up to two hours later.

Results: A positive test is an absorbance value that is >0.20 above the absorbance observed in the negative controls (sterile uninoculated TPGY broth or CMM).

D. **Confirmation of positive ELISA samples.**
The ELISA is used for screening culture media that may contain type A, B, E, and/or F botulinal toxins. Samples that are positive using the ELISA must be confirmed using the mouse bioassay.

Flow Diagram for Amp-ELISA

Day 1

Coat microtiter plates with capture IgG and store overnight at 4°C.

Day 2

1. Wash plates, block, put on toxic samples and controls, 2 hr incubate.

2. Wash, put on biotinylated IgG's, 1 hr incubate.

3. Wash, put on the Extravidin conjugate, 1 hr incubate.

4. Wash, put on Gibco substrate, 12.5 min incubate

5. Put on Gibco amplifier, 2-10 min incubate.

6. Read plates on microplate reader

References

1. Ferreira, J L., Maslanka, S, Johnson, E., and Goodnough, M. 2003. Detection of botulinal neurotoxins A, B, E, and F by amplified enzyme-linked Immunosorbent assay: collaborative study. *JAOAC International* **86**:314-331.

2. Solomon, H. and Lilly, T. 2001. FDA Bacteriological Analytical Manual. Chapter 17, *Clostridium botulinum.*

3. Ferreira, J.L. 2001. Comparison of amplified ELISA and mouse bioassay procedures for determination of botulinal toxins A, B, E, and F. *JAOAC International* **84**:85-88.

A modification of the method described above is available in Laboratory Information Bulletin (LIB) No. 4292. The LIB describes a modification that uses digoxigenin labeled IgGs to detect type A, B, E, and F botulinal toxins. The digoxigenin label substitutes for the biotin label in the amplified ELISA and is detected using an anti-digoxigenin horse radish peroxidase conjugate and TMB substrate.

IV. **Detection of Type A, B, E, and F *Clostridium botulinum* Toxins Using Digoxigenin-labeled IgGs and the ELISA (DIG-ELISA).** Contact J. L. Ferreira (FDA) 404 253-2216, S. Sharma (FDA) 301 *436-1570*. S. Maslanka (CDC) 404 639-0895, or J. Andreadis (CDC) for questions regarding this method.

This method is a modification of the amplified-ELISA (amp-ELISA). Digoxigenin-labeled antitoxin IgG's are substituted for biotin-labeled IgG's and anti-digoxigenin horse radish peroxidase conjugate (HRP) is substituted for the streptavidin-alkaline phosphatase used in the amp-ELISA. An appropriate substrate (TMB) is used for the HRP enzyme. The A, B, E, and F botulinal toxins are detected at approximately 10 MLD/mL (0.12-0.25 ng/mL). Toxic cultures may be more antigenic than purified toxins and the level of detection using the DIG-ELISA may be more sensitive than the mouse bioassay. Both TPGY and CMM are tested since more toxin may be generated in one medium compared to the other and the confirmatory mouse bioassay also utilizes these media. Very toxic cultures (greater than approximately 10,000 MLD/mL) may give a positive absorbance for more than one toxin type in the amp-ELISA as well as the DIG-ELISA (crossing between types). Generally, a 10-fold dilution will show that the true toxin type will have a very high absorbance and the crossing type will have a negative absorbance. In either case the toxic sample must be confirmed using the mouse bioassay.

A. **Equipment and Materials**

1. Microplate, Dynex Immulon ll U-bottom, cat. No. 3655

2. Microtiter pipettors to deliver from 0.1- 2.0, 2-20, and 50-200 µl.

3. Multichannel pipettor, 8 or 12 place 50-200 µl

4. Pipets, disposable 1,5,10 ml

5. Glass test tubes 13X100 mm, 15X150 mm

6. Incubator, 35°C

7. Refrigerated centrifuge

8. Microplate washer

9. Microplate shaker

10. Microplate reader (read 450 nm)

11. Microtiter plate seals

12. Multichannel pipet reservoirs

B. Media (/food/laboratory-methods/media-index-bam) and Reagents (/food/laboratory-methods/reagents-index-bam)

1. Tryptone-peptone-glucose-yeast extract broth (TPGY).

2. Cooked meat medium (CMM).

3. 0.05M bicarbonate buffer: 0.8g Na_2CO_3 + 1.47g $NaHCO_3$ in 500 ml distilled H_2O, pH 9.6. Capsules to prepare 100 ml volume are available from Sigma.

4. 1% Casein buffer: Add 10.0g vitamin-free casein (Research Organics) + 7.65g NaCl, 0.724g Na_2HPO_4 (anhydrous), 0.21g KH_2PO_4 to 900 ml H_2O, and 3 ml of 1 M NaOH. Heat with stirring to ~ 80°C to dissolve casein. Check pH and adjust to 7.9 with 1 M NaOH, q.s. to 1 liter. Sterilize at 121°C for 20 min. Final pH is ~7.4-7.6. Casein blocker ready to use product is available from Pierce that gives slightly lower absorbance values than in-house prepared casein buffer. (SRL, Atlanta, GA).

5. Goat type A, B, E, or F digoxigenin-labeled antitoxin (SRL, Atlanta, GA).

6. Phosphate buffered saline with 0.005% Tween 20 wash buffer (PBST).
1.2 g Na_2HPO_4 (anhydrous), 0.22g $NaH_2PO_4.H_2O$, 8.5g NaCl per liter distilled H_2O. Adjust pH to 7.5 Add 50 μl

of Tween 20/L PBS. Sterilize at 121°C for 20 min. 10 × PBST: 12.0g Na_2HPO_4 (anhydrous), 2.2g $NaH_2PO_4.H_2O$, 85.0g NaCl per liter distilled H_2O. Adjust pH to 7.5 Add 500 μl of Tween 20/L PBS. 1 × PBST is then prepared by adding 100 ml of 10X PBST to 900 ml of distilled H_2O and mixing before use. 10X PBS is available commercially from GibCo.

7. Anti-digoxigenin HRP poly conjugate (Roche Applied Science).

8. Tetra methyl benzidine (Ultra-TMB) (Pierce).

9. 0.5 M H_2SO_4.

10. Botulinal complex toxin standards A, B, E, and F. (Metabiologics Inc., Madison, WI)

C. **DIG-ELISA Procedure**

1. **Preparation of samples.**

   a. **Cultural sample preparation.** Food samples or anaerobic isolates picked from agar plates are inoculated into TPGY (**without trypsin**) and CMM as recommended in Chapter 17 of the Bacteriological Analytical Manual (2001). TPGY broth and cooked meat media are incubated for 5 days at 26°C and 35°C respectively. Cultures are centrifuged at 7,000 × g and 4°C for 30 min, supernatant pH is adjusted to 7.4-7.6 using 1 N NaOH or 1N HCl. Samples and controls are analyzed **in duplicate** for TPGY and for CMM. Analyze undiluted and 1:5 dilutions of each culture supernatant. 1:5= 0.2 ml culture + 0.8 ml casein buffer.

b. **Food sample preparation.** If a food has a liquid packing medium, the liquid may be removed, centrifuged as above to remove solids and/or fats and the supernatant/ aqueous layer directly analyzed by ELISA after pH adjustment to 7.4-7.6. If the food is a solid or semi-solid, the toxin must be extracted. An equal amount of food (20 g) and casein buffer (20 mL) are mixed by grinding with a mortar and pestle or by other means to mix the food and buffer. The food-buffer slurry (1:2 dilution) is centrifuged at 7,000 × g for 30 min at 4°C. The aqueous supernatant is removed and adjusted to pH 7.4-7.6 if necessary using 1 N NaOH or 1 N HCl. Some foods such as Honey may also require dilution to remove ELISA inhibitors. Honey has previously been tested at a 1:5 dilution with satisfactory results. Normal food that does not contain botulinal toxin can be spiked with known standard toxin(s) at 2ng toxin/mL (~100 MLD/mL) of the food extract in casein buffer to monitor the possible inhibition of the ELISA by the food. Botulinal neurotoxin standards were diluted in casein buffer and used as controls or for spiking foods prior to analysis.

2. **Preparation of microtiter plates.** Coat each well of the microtiter plate with 100 μl of appropriate dilution of goat type A, E, or F or rabbit type B antitoxin diluted in bicarbonate buffer. Prepare the number of needed microtiter plate wells to test the sample. Dilute the stock antitoxins according to the accompanying directions. Store plate with coating buffer overnight at 4°C with

plastic seal cover on top of plate to prevent evaporation.

3. **ELISA analysis of samples.**

   a. Remove plate from 4°C storage and wash plate 5 times in PBST with 45 second hold between each aspiration. Use a commercial plate washer or other mechanical device; avoid using a squeeze bottle to wash.

   b. Block plate in casein buffer with by filling all wells to the top of the plate (~300 µl/well) and incubate for 60-90 min at 35°C. Prepare the sample and control dilutions while the plate is being blocked.

   Negative controls: Duplicate wells are tested with all reagents except toxin (pH adjusted undiluted sterile CMM and TPGY broth if used and casein control). Casein buffer control is used as a system control.

   Positive controls: Duplicate wells are tested using standard toxins type A, B, E, and F diluted in pH adjusted sterile TPGY and CMM (if used) at a concentration of 2 ng/mL. The LD_{50}/ng will vary depending on toxin type.

   ELISA Food Inhibition controls: Type A, B, E, and F neurotoxins can be used to spike a food at 2 ng/mL of the supernatant obtained from the food-casein buffer slurry. Duplicate wells are tested for each toxin type. Results are compared to the positive control that consists of toxin spiked into casein to demonstrate if the product inhibits the

ELISA. The product may be diluted further to remove inhibitory substances but will lower the sensitivity of the test.

c. Wash the blocked plate as above and then add the toxic samples and controls (100 µl/well). Work from the left side of the plate to the right side when adding the reagents.

d. Incubate toxin-containing samples and controls for 2 hr. at 35°C. Prepare the type A, B, E, and F digoxigenin-labeled antibody reagents according to directions while incubating the samples. Do not make more than you need!

e. Wash plate 5 times in PBST as above.

f. Add the diluted digoxigenin-labeled goat antibody (100 µl/well) and incubate for 60 min at 35°C.

g. Wash plate 5 times in PBST as above.

h. Add the anti-digoxigenin poly HRP conjugate diluted 1:5,000 in casein buffer (100 µl/well), and incubate for 60 min at 35°C.

i. Wash 5 times in PBST then tamp the plate several times on a paper towel to remove any residual wash buffer.

j. Add 100 µl of the TMB (substrate at room temperature) solution, incubate 20-30 min at 35°C. Positive sample wells will begin to turn a blue-green color. High toxin samples will develop color within a few minutes. The analysis can be stopped with 100 µl of stop reagent at any time (within 20-30 min) after addition of the substrate when

positive controls give appropriate sensitivity (absorbance ≥ 1.0) and negative controls are acceptable (absorbance not greater than ~ 0.39). The plate should be taken to the plate reader immediately after addition of the stop solution. Measure absorbance at 450 nm on microplate reader.

Results: A positive test is an absorbance value that is >0.20 above the absorbance observed in the negative controls (sterile uninoculated TPGY broth or CMM or negative food sample). As in any ELISA, higher background absorbance will result if plates are insufficiently washed.

D. **Confirmation of positive ELISA samples.** The DIG-ELISA was designed for screening TPGY and CMM culture media that may contain type A, B, E, and/or F botulinal toxins. Some food matrices may be inhibitory to the test or may generate false positive results. Samples that are positive or are inhibitory to the DIG-ELISA test must be confirmed using the mouse bioassay.

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1. Ferreira, J L., Maslanka, S, Johnson, E., and Goodnough, M. 2003. Detection of botulinal neurotoxins A, B, E, and F by amplified enzyme-linked Immunosorbent assay: collaborative study. *JAOAC International* **86**:314-331.

2. Solomon, H. and Lilly, T. 2001. FDA Bacteriological Analytical Manual. Chapter 17, *Clostridium botulinum.*

3. Ferreira, J.L., Maslanka, S., Andreadis J. 2002. Detection of type A, B, E, and F *Clostridium botulinum* toxins using digoxigenin-labeled

IgGs and the ELISA. FDA/ORA Laboratory Information Bulletin Vol. 18, Number 10, 4292:1-10.

Flow Diagram for DIG-ELISA

Day 1

Coat microtiter plates with capture IgG and store overnight at 4°C.

Day 2

1. Wash plates, block, put on toxic samples and controls, 2 hr incubate.
2. Wash, put on digoxigenin-labeled IgG's, 1 hr incubate.
3. Wash, put on the anti-digoxigenin HRP conjugate, 1 hr incubate.
4. Wash, put on TMB substrate, 20-30 min incubate.
5. Stop the reaction with stop reagent.
6. Measure absorbance on plates with microplate reader at 450 nm.

V. **Specific Detection of Clostridium botulinum Types A, B, E, and F Using the Polymerase Chain Reaction (PCR)** For additional information on this PCR method, contact Kathy E. Craven or Joseph L. Ferreira at FDA, ORA, Southeast Regional Laboratory, 60-8th Street, N.E., Atlanta, GA 30309. Telephone: (404) 253-1200; FAX: (404)253-1210.

Clostridium botulinum organisms generally produce one of four neurotoxin types (A, B, E, and F) associated with human illness. Neurotoxin type determination is important in determining the identification of the bacterium. A PCR method was developed to identify 24 hour botulinal cultures as potential type A, B, E and F neurotoxin producers as well as culture of other clostridial species which also produce botulinal neurotoxins. Components of the PCR

and amplification conditions were adjusted for optimal amplification of toxin gene target regions enabling the simultaneous testing for types A, B, E, and F in a single thermal cycler. Each primer set was specific for its corresponding toxin type. Additionally, a DNA extraction procedure was included to remove inhibitory substances that may affect amplification. This procedure is rapid, sensitive, and specific for the identification of toxigenic *C. botulinum*.

Because of the severity of neuroparalytic illness caused by botulinal neurotoxin, a rapid diagnosis for the specific toxin type is necessary during illness outbreaks suspected of being foodborne. The PCR technique has also been used to detect multiple botulinal toxin-producing types within a single PCR assay (4,6). The PCR assay for the toxin gene type is determined after a 24-hour anaerobic culture to obtain vegetative cells. ELISA procedures may require up to five days of culture growth before toxin is detected (5,9). The PCR method may also be used in conjunction with the mouse bioassay to determine toxin type. For example, a culture that is PCR positive for the type A toxin gene would require mouse protection/testing confirmation only for toxin type A.

A. **Equipment and Materials**

    1. Programmable automatic thermocycler

    2. Horizontal gel electrophoresis apparatus

    3. Electrophoresis constant-voltage power supply

    4. Heating plate

    5. Incubators, 35°C

    6. Water bath, 37°C and 60°C

    7. Freezer, -20 and -70°C

8. Speed Vacuum, optional

9. Microwave

10. Sterile disposable inoculating loops

11. Microcentrifuge tubes, 1.5 and Thin Walled PCR reaction tubes, 0.2 ml or 0.5 ml

12. Variable digital micropipettors (e.g., 0.5-20 µl, 20-200 µl, 100-1,000µl)

13. Aerosol-resistant pipet tips

14. Microcentrifuge

15. UV transilluminator

16. Polaroid camera and Polaroid film 3000 ISO or comparable Gel Documentation System

B. Media (/food/laboratory-methods/media-index-bam) and Reagents (/food/laboratory-methods/reagents-index-bam)

Molecular biology grade reagents are recommended and are available from various manufacturers.

1. Tryptone-peptone glucose yeast extract broth (TPGY).

2. Phosphate-buffered saline, pH 7.4 (PBS)

3. Tris EDTA, pH 8.0 (1X TE). 10mM Tris-HCL, 1mM EDTA, pH 8.0 in distilled water

4. Proteinase K- 10 mg Proteinase K/ml 1× TE

5. Lysozyme-10 mg Lysozyme/ml 1 × TE

6. 3 M Sodium Acetate, pH 5.2

7. 95% ethanol

8. 2'-Deoxynucleoside-5'-triphosphates (dATP, dCTP,

dGTP, dTTP); stock solution 2.5 mM of each dNTP

9. *Taq* DNA polymerase (available from various vendors) or Amplitaq® (Perkin-Elmer)

10. 10 × Reaction Buffer B-500mM KCl, 100 mM Tris-HCl (pH 9.0 at 25°C), 1.0 % Triton X-100

11. 15 mM MgCl$_2$

12. *Clostridium botulinum* neurotoxin oligonucleotide primers types A, B, E, and F, 10 µM stock solutions (2).

13. Light mineral oil, optional

14. Sterile deionized water, RNase and DNase free

15. 10× TBE (0.9 M Tris-borate, 0.02 M EDTA, pH 8.3)

16. Agarose (nucleic acid electrophoresis grade)

17. Ethidium bromide solution, 10 mg/ml

18. 6× sample loading buffer

19. DNA molecular weight markers (e.g., 123 bp ladder or 100 bp ladder)

C. **Procedure for amplification of *C. botulinum* neurotoxin A, B, E, and F gene fragments from presumptive *C. botulinum* isolates using TPBY enrichment broth**

Food sample preparation and enrichment (Chapter 17, Part l Mouse Bioassay, Section D).

1. **DNA isolation Procedures.** Boil sterile 10 ml portions of Tryptone-Peptone-Glucose-Yeast Extract Broth (TPGY) in a water bath for 10 min and quickly cool to room temperature just prior to use. Inoculate TPGY with presumptive *C. botulinum* isolates using a

disposable sterile inoculating loop and incubate overnight at 35° C. Remove a 1.4 ml aliquot from each of the cultures and dispense into separate sterile micro-centrifuge tubes. Centrifuge at 14,000 × g for 2 min and discard supernatant. Wash the bacterial pellets in 1.0 ml PBS, pH 7.4 and centrifuge at 14,000 × g for 2 min. Discard supernatant and resuspend pellets in 400 µl PBS and 100 ml of 10 mg lysozyme/ml 10mM Tris, 1 mM EDTA, pH 7.4 (TE). Incubate for 15 min at 37° C in a water bath, inverting tubes every 5-7 min during incubation. Add 10 µl of 10 mg Proteinase K/ml TE to suspensions and incubated for 1 h in a 60° C water bath. Invert tubes every 10-15 min during the incubation period. Boil suspensions for 10 min in a water bath and centrifuge for 2 min at 14,000 rpm. Transfer supernatants to sterile 1.5 ml micro-centrifuge tubes. Add 50 µl aliquot of 3 M Sodium acetate and 1.0 ml of 95% ethanol to supernatants, mix by inversion, and cool at -70 °C (or -20°C) for 30 min. Centrifuge the ethanol-salt preparations at 14,000 rpm. Discard supernatants and dry pellets using a DNA Speed-Vacuum (Savant Instruments, Inc., Holbrook, NY). Re hydrate pellets in 200-µl sterile TE buffer and store immediately at -20° C until PCR analysis is performed.

2. **Alternative DNA isolation/preparation procedures.** Cell lysis by boiling can also be performed to simplify the procedure. *C. botulinal* cultures are grown 24 hours as previously described. Remove a 1.4 ml aliquot and centrifuge at 14,000 × g for 2 min. Boil the suspension in a water bath for 10 min and centrifuge at 14,000 × g for 2 min to remove cell debris. Remove the

supernatants and place into a sterile microcentrifuge tube. Store at -20°C until PCR analysis is performed. Commercial DNA extraction kits such as Gene Clean II (BIO 101,Inc., La Jolla, CA) and S&S Elu-Quick (Schleicher & Schuell, Keene, NH) may be used if the cells are sufficiently lysed. Manufacturers' protocol supplied with kits are followed. The method used for lysis of gram positive organisms prior to extraction of the DNA for PCR is important. Unless DNA concentrations are determined before PCR analysis, it may be necessary to test dilutions of the DNA sample to avoid false negative results caused by too little or too much DNA when using commercially available kits. We recommend the use of no more than 344 ng of total DNA be used for the PCR analysis.

Note: DNA purification before amplification is recommended to reduce the possibility of inhibitory substances in cultures from affecting the PCR and to increase the concentration of target DNA. Purification of DNA removes inhibitory substances that may affect PCR amplification. Simple boiling of the cell culture may not remove all inhibitors from the PCR DNA preparation for all cultures. No PCR inhibition was observed due to the TPGY medium itself. The use of the described extraction procedure that incorporates Proteinase K and lysozyme consistently lysed *C. botulinum* cells (2). The amount of isolated DNA yielding positive results using this amplification method ranged from approximately 0.34 ng- 5,160 ng DNA per 100-µl total volume PCR reaction. Using DNA concentrations outside this range may result in false negative results.

This method is rapid and reliable for the identification of type A, B, E and F toxin-producing clostridial strains. PCR results for typing clostridial toxin genes were obtained in approximately 4 hours following a 24-hour incubation of the culture. This method is not limited by culture production of the neurotoxin which requires up to five days incubation prior to analysis by ELISA or the mouse bioassay (3,5). The PCR products also can be toxin gene typed or confirmed by using type-specific oligonucleotide or polynucleotide DNA probes.

Oligonucleotide Primers. Desalted oligonucleotide primers are obtained from commerical suppliers. Primers were derived from published DNA sequences for *C. botulinum* structural genes encoding types A, B, E, and F neurotoxins (1, 3, 7, 8). The forward (F) and reverse (R) PCR primer sequences are:

Type A
F 5' -GTG ATA CAA CCA GAT GGT AGT TAT AG -3'
R 5' -AAA AAA CAA GTC CCA ATT ATT AAC TTT -3'

Type B
F 5' -GAG ATG TTT GTG AAT ATT ATG ATC CAG -3'
R 5'- GTT CAT GCA TTA ATA TCA AGG CTG G -3'

Type E
F 5'- CCA GGC GGT TGT CAA GAA TTT TAT -3'
R 5'- TCA AAT AAA TCA GGC TCT GCT CCC -3'

Type F
F 5'-GCT TCA TTA AAG AAC GGA AGC AGT GCT-3'
R 5'- GTG GCG CCT TTG TAC CTT TTC TAG G -3'

PCR reaction preparation. Primer sets for each of the types are used in separate PCR reactions. PCR reactions are performed in a 100 μl volume mixture containing , 1 × PCR buffer [10 mM Tris-HCl pH 9.0, 50 mM KCl, and 0.1% Triton X-100], 2.5 mM $MgCl_2$, 0.5 μ'M concentration of each primer set (A, B, E, or F), 200 μM concentration of each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 2.5 U *Taq* DNA polymerase, and 2 μl of sample DNA. If necessary add approx. 50-70 μl of sterile mineral oil. Thermal cyclers equipped with heated covers will not require the addition of a mineral oil overlay. If PCR reaction volumes are decreased to 50 μl, the amount of template should be decreased to 1.0 μl.

Note: It is recommended to add sample DNA to the PCR reaction mixture last in order to decrease potential contamination of PCR reagents. Positive and negative controls should be included in each analysis. Negative controls containing all of the reagents but lacking template DNA processed as described above are used to monitor for contamination with *C. botulinum* amplicons.

Temperature cycling. PCR conditions for simultaneous amplification of toxin gene fragments A, B, E, and F are:

One cycle at 95°C for 5 min
Thirty cycles of 94 °C for 1 min (denaturation)
60°C for 1 min (annealing)
72°C for 1 min (extension)
Final incubation of 72 °C for 10 min
Holding temperature of 4°C

Multiplex PCR for the amplification of A and E or B and F toxin gene fragments has been performed successfully using these primers but with lower PCR product yields (4). These four primer pairs can not be used together in one multiplex reaction because the primers are incompatible.

Agarose gel analysis of PCR products. Prepare a 1.2-1.5 % agarose gel in 0.5 × TBE containing 0.5 µg ethidium bromide/ml agarose. Agarose may be melted in 0.5 × TBE using a microwave. Cast gel and allow to solidify. Mix 10 µl portions of PCR products with approximately 2.0 µl 6× gel loading dye and load onto gel submerged in 1 × TBE. An appropriate molecular weight marker must be included on each gel in order to determine the approximate molecular weight of PCR products. Molecular weight markers should contain fragments which bracket the target sequence size. Apply a constant voltage of 10 V/cm and allow amplified fragments to migrate until appropriate band separation is achieved.

A short-wave UV light is used to visualize bands relative to the molecular weight marker. Predicted fragment lengths for each toxin gene fragment are: Type A, 983-bp; Type B, 492-bp; Type E, 410-bp, and Type F, 1137-bp. Photographs of the gels are used to document the results using either a polaroid camera or a comparable gel documentation system.

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Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998. Chapter 17.

Name: Ascus Biosciences Inc.

Customer: Cindy Hoppe

Address: 6450 Lusk Blvd. Suites E109/209
San Diego, CA
92121
USA
877-696-8945

Order ID:

Report ID:

Date Received: 9/19/2019 10:25:27

Reported: 9/25/2019 14:43:41

P.O. #: N/A

Page: 1 of 1

Report of Results

Analysis Date: 2019/09/19 **Receiving Temperature:** ambient **Sample Condition:** Okay

Description: 18-0202-041-P22

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

Analysis Date: 2019/09/19 **Receiving Temperature:** ambient **Sample Condition:** Okay

Description: 18-0202-001-P73

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

Analysis Date: 2019/09/19 **Receiving Temperature:** ambient **Sample Condition:** Okay

Description: 18-0202-001-P79

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

EASY SCAN

Personalized PDF Catalog

Catalogue Generated March 27, 2019

Acetic acid glacial 99.7-100.5% USP, FCC, ACS,

(b) (4)

(b) (4)

Danger

Synonyms: Ethanoic acid, Glacial acetic acid, Methanecarboxylic acid, Vinegar acid

Only Class 3 Solvents are likely to be present. Residual concentration of all Class 3 Solvents is <0.5%.

Elemental Impurities (USP 232, EP 5.20) - Information on elemental impurities for this product is available the associated Product Regulatory Data Sheet and elemental impurity profile report.

Caution: Preserve in tight containers, and store at room temperature

Formula: H_3CCOOH
MW: 60,05 g/mol
Boiling Pt: 118 °C (1013 hPa)
Melting Pt: 17 °C
Density: 1,05 g/cm³ (20 °C)
Flash Pt: 39,4 °C (closed cup)
Storage Temperature: Ambient

MDL Number: MFCD00036152
CAS Number: 64-19-7
EINECS: 200-580-7
UN: 2789
ADR: 8,II
REACH: 01-2119475328-30
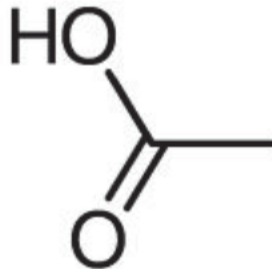
Specification Test Results

USP - Assay (CH_3COOH)	99.7 - 100.5 %
USP - Chloride (Cl) (USP)	Passes Test
USP - Congealing Temperature	>= 15.6 °C
USP - Heavy Metals (as Pb)	<= 0.5 ppm
USP - Identification A	Passes Test
USP - Identification B	Passes Test
USP - Readily Oxidizable Substances	Passes Test
USP - Sulfate (USP)(SO_4)	Passes Test
USP - Limit of Nonvolatile Residue	<= 0.005 %
FCC - Assay (CH_3COOH)	99.7 - 100.5 %
FCC - Identification A	Passes Test
FCC - Identification B	Passes Test
FCC - Lead (Pb)	<= 0.5 mg/kg
FCC - Limit of Nonvolatile Residue	<= 0.005 %
FCC - Readily Oxidizable Substances	Passes Test

FCC - Solidification Point	>= 15.6 °C
ACS - Assay (by GC, corrected for water)	>= 99.7 %
ACS - Acetic Anhydride ($(CH_3CO)_2O$)	<= 0.01 %
ACS - Chloride (Cl)	<= 1 ppm
ACS - Color (APHA)	<= 10
Dilution Test	Passes Test
ACS - Iron (Fe)	<= 0.2 ppm
ACS - Heavy Metals (as Pb)	<= 0.5 ppm
ACS - Substances Reducing Dichromate	Passes Test
ACS - Substances Reducing Permanganate	Passes Test
ACS - Sulfate (SO_4)	<= 1 ppm
ACS - Titrable Base (meq/g)	<= 0.0004
Copper (Cu)	<= 0.00001 %
Nickel (Ni)	<= 0.00001 %
Residue after Evaporation	<= 0.001 %

ORDER

(b) (4) Catalog Number	Unit	Price	Quantity	Size	Packaging	Supplier No.
(b) (4)	Case of 6 (500ml)	(b) (4)	🚫	500 mL	Clear Glass Bottle, PVC Coated	(b) (4)
(b) (4)						
(b) (4)	Case of 6 (2,500 ,ml)	(b) (4)	🚫	2.5 L	Clear Glass Bottle, PVC Coated	(b) (4)
	Each(2,500,ml)	(b) (4)	🚫			
(b) (4)						

In order to process your orders without delay, we request that you provide the required business documentation to purchase this product.

To order chemicals, medical devices, or other restricted products please provide identification that includes your business name and shipping addr (b) (4) referencing your (b) (4) account number . Acceptable forms of identification are:

State issued document with your organization's Federal Tax ID Number

State issued document with your organization's Resale Tax ID Number

City or County issued Business License

State Department of Health Services License

Any other ID issued by the State that includes the business name and address

* Please note if your account is within the State of California two of these pieces of identification will be required. (b) (4) will not lift restrictions for residential shipping addresses.

Specification for Ammonium Chloride, Granular, FCC (A1167)

Item Number	A1167
Item	Ammonium Chloride, Granular, FCC
CAS Number	12125-02-9
Molecular Formula	NH_4Cl
Molecular Weight	53.49
MDL Number	
Synonyms	

Test	Specification	
	Min	Max
ASSAY (DRIED BASIS)	99.0 %	
LEAD (Pb)		4 mg/kg
LOSS ON DRYING		0.5 %
IDENTIFICATION	TO PASS TEST	
RETEST DATE		

Ammonia Solution

(b) (4)

PRODUCT INFORMATION

AMMONIA SOLUTION FG

PRODUCT DESCRIPTION

Ammonia Solution FG is a water white solution of anhydrous ammonia in water that meets food grade requirements and reference 21 CFR 184.1139.

CAS No. 1336-21-6

SYNONYMS

Ammonium hydrate

USES AND APPLICATIONS

Ammonia Solution FG may be used to replace anhydrous ammonia in most applications.

Product is designed for use in food and fermentation applications.

TYPICAL VALUES

Appearance:	Clear, Colorless Liquid
Odor:	Ammonia
Ammonia as NH_3 (wt.%):	27.0 – 30.0
Lead (ppm):	0.5 max
Nonvolatile Reside (wt.%):	0.02 max
Readily Oxidizable Substances:	Passes FCC Test

SHELF LIFE: Shelf life stability is dependent on temperature. At room temperature, the shelf life is 90 days.

PRECAUTIONS

Product safety information and handling precautions are contained on the product label and Safety Data Sheet (SDS).

READ AND UNDERSTAND LABEL AND SAFETY DATA SHEET BEFORE PRODUCT USE.

NH0028
8/1/2018
Rev: 21

(b) (4)

Biotin

Specification for Biotin, Powder, FCC (BI115)

Item Number	BI115
Item	Biotin, Powder, FCC
CAS Number	58-85-5
Molecular Formula	$C_{10}H_{16}N_2O_3S$
Molecular Weight	244.31
MDL Number	
Synonyms	Vitamin H

Test	Specification	
	Min	Max
ASSAY ($C_{10}H_{16}N_2O_3S$)	97.5-100.5 %	
MELTING RANGE	229 - 232 C (dec	
OPTICAL ROTATION	+89 to +93	
LEAD (Pb)		2 mg/kg
IDENTIFICATION		TO PASS TEST
RETEST DATE		

Cerelose® Dextrose M Non-GMO

technical specification

CERELOSE® Dextrose M NON-GMO IP 02001090

CERELOSE® Dextrose 02001090 is a general purpose crystalline monohydrate dextrose suitable for most food, beverage, and industrial uses. This product is produced under

Chemical and Physical Properties

	Min.	Max.
Moisture %	8.0	9.0
Dextrose Equivalent	99.5	-
SO₂, ppm	-	<10
Dextrose, % d.b.	99.5	-
Ash, % d.b.	-	0.1
Solution Color		Passes test
Apparent Starch		Passes test

Physical Appearance

	Typical
Color	White
Form	Powder

Screen Test

	Typical
On USS 20 mesh, %	<1
On USS 100 mesh, %	<60

Microbiological Limits

	Max.
Standard Plate Count, cfu/g	100
Yeast, cfu/g	25
Mold, cfu/g	25
Salmonella/10 g	Negative
Coliforms, MPN/g	3

Nutritional Data/ 100g

	Typical
Calories	362
Calories from Fat	0
Total Fat, g	0
Cholesterol, mg	0
Sodium, mg	0
Total Carbohydrate, g	90.5
Dietary Fiber, g	0
Total Sugars**, g	90.5
Added Sugars, g	0
Other Carbohydrate, g	0
Protein, g	0
Vitamin D, mcg	0
Calcium mg	0
Iron, mg	0
Potassium, mg	0
Ash, g	<0.1*

* Not present at level of quantification.
** "Total Sugars" in this product may contribute to "Added Sugars" for nutrition labeling purposes in the final consumer product.

Certification

Kosher Pareve
Halal

Packaging and Storage

Bags
Product should be stored in a clean, dry area, not exposed to prolonged high (> 90°F / 32°C) temperature.

Shelf Life

3 years, provided product is stored the original container, well-closed in a cool, dried place free from humidity, dust, or foreign contamination.

Regulatory Data

Source	Corn (IP-TrueTrace™)
CAS No.	50-99-7

United States

Meets FCC (Food Chemical Codex) requirements.

Standard of Identity	21 CFR 168.111
GRAS Affirmation	21 CFR 184.1857
Labeling	Dextrose or Dextrose monohydrate

Canada

Standard Food	CFDA Regulation
Standard of Identity	B.18.015
Labeling	Dextrose or Dextrose monohydrate

Features and Benefits

TrueTrace™ certified non-GM.
Dry crystalline powder, Free flowing.
Mild sweetness
Bulking, Carrying
Highly fermentable

Specifications for Salt

Ingredient:	Sodium Chloride
Chemical Nomenclature:	NaCl
Specifications:	Feed/Food Grade or FCC
Moisture:	≤ 1.5% by LOD
Purity:	≥ 95%

Confidential Manufacturing Information

The raw materials used in the manufacture of *Clostridium beijerinckii* ASCUSDY20 are listed in Table 1 below. Specifications for the raw materials are provided in Appendices 009A to 009S.

Table 1: Raw Materials and Processing Aids Used in the manufacture of *Clostridium beijerinckii* ASCUSDY20			
Material	Function	Regulatory Status	Grade
Acetic Acid Glacial	Nutrient	GRAS substance for use as a general purpose food additive (21 CFR 582.1005)	FCC
Ammonium Chloride	Nutrient	AAFCO OP ingredient definition (mineral product) 57.265	FCC
Ammonium Hydroxide	Nutrient	GRAS substance for use as a general purpose food additive (21 CFR §582.1139)	FCC
Biotin	Nutrient	GRAS substance for use as a nutrient and/or dietary supplement (21 CFR §582.5159)	FCC
Condensed Fermented Corn Extractives (Solulys 095K Corn Steep Powder)	Nutrient	AAFCO OP ingredient definition (corn product) 48.24	Feed grade
Dextrose Monohydrate	Nutrient	Common ingredient (*e.g.*, 21 CFR §168.111; 21 CFR §184.1857)	FCC
Dipotassium Phosphate	Buffering agent	GRAS substance for use as a sequestrant (21 CFR §582.6285)	FCC
Ferrous Sulfate Heptahydrate	Nutrient	AAFCO OP ingredient definition (mineral product) 57.83; GRAS substance for use as a nutrient and/or dietary supplement (21 CFR 582.5315)	Feed grade
Folic Acid	Nutrient	GRAS substance for use as a nutrient and/or dietary supplement (21 CFR 172.345)	FCC
L-Cysteine Hydrochloride	Nutrient	GRAS substance for use as a nutrient and/or dietary supplement (21 CFR 582.5271)	FCC
Magnesium Sulfate Heptahydrate	Nutrient	GRAS substance for use as a nutrient and/or dietary supplement (21 CFR §582.5443) AAFCO OP ingredient definition (mineral product) 57.88	FCC
Manganese Sulfate Monohydrate	Nutrient	GRAS substance for use as a nutrient and/or dietary supplement (21 CFR §582.5461) and trace mineral (21 CFR §582.80)	FCC
Modified Starch; CAPSUL® 06670103	Carrier	GRAS substance for use as a general purpose food additive 21 CFR 172.892	Food grade

Monopotassium Phosphate	Buffering agent	Permitted for use as a food additive in frozen FCC eggs (21 CFR §160.110) – safety for use in feed assessed by ASCUS (Appendix 009N2)	FCC
Polyglycerol polyethylene-polyoxypropylene block copolymer	Anti-foaming agent	Acceptable for use as an anti-foaming agent for the production of enzymes and DFMs in accordance with the letter issued by the FDA to the Enzyme Technical Association (ETA, Appendix 009O2)	Food grade
Sodium Chloride	Nutrient	AAFCO OP ingredient definition (mineral product) 57.31	Food grade
Thiamine Hydrochloride	Nutrient	GRAS substance for use as a nutrient and/or dietary supplement (21 CFR §582.5875) AAFCO OP ingredient definition (recognized vitamin ingredients) 90.25	FCC
Yeast Extract (Amberex 1003 AG)	Nutrient	Yeast extract obtained by mechanical rupturing of cells is accepted for use in feed (AAFCO OP 96.11); use of autolysis in the production of the extract is not expected to introduce any different substances and should yield a product with equivalent composition – history of use in food (*e.g.*, FCC monograph established Appendix 009S2)	Food grade
Yeast Extract (Procelys Springer 0251/ 0-MG-L)	Nutrient	Yeast extract obtained by mechanical rupturing of cells is accepted for use in feed (AAFCO OP 96.11); use of autolysis in the production of the extract is not expected to introduce any different substances and should yield a product with equivalent composition – history of use in food (e.g., FCC monograph established Appendix 009S2)	Food grade

Abbreviations: OP – Official Publication; FCC – Food Chemicals Codex; USP – United States Pharmacopoeia

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Confidential Detailed Manufacturing Summary of *Clostridium beijerinckii* ASCUSDY20 SDP

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1   Overview

Clostridium beijerinckii ASCUSDY20 SDP (Spray Dried Powder) is produced through a series of processes: Fermentation, Centrifugation and Spray Drying. A process diagram of the production of *Clostridium beijerinckii* ASCUSDY20 SDP is below (Appendix A). The strain (*Clostridium beijerinckii* ASCUSDY20) is an obligate anaerobic spore-forming *Clostridium beijerinckii* bacterium, that is produced by dextrose fed-batch anaerobic fermentation in such a way as to induce sporulation of the strain.

(b) (4)

to yield the *Clostridium beijerinckii* ASCUSDY20 SDP notified substance. *Clostridium beijerinckii* ASCUSDY20 SDP is then packaged into moisture barrier mylar bags, heat sealed, and stored at less than 25°C.

2   Master Cell Bank / Working Cell Bank

(b) (4)

3   Fermentation

(b) (4)

(b) (4)

(b) (4)

4 Biomass Harvest by Centrifugation

(b) (4)

5 Spray Drying

(b) (4)

(b) (4)

Appendix A. Process Diagram of the Production of *Clostridium beijerinckii ASCUSDY20 SDP*

Clostridium beijerinckii ASCUSDY20 Manufacturing Process

10 Jan 2020

(b) (4)
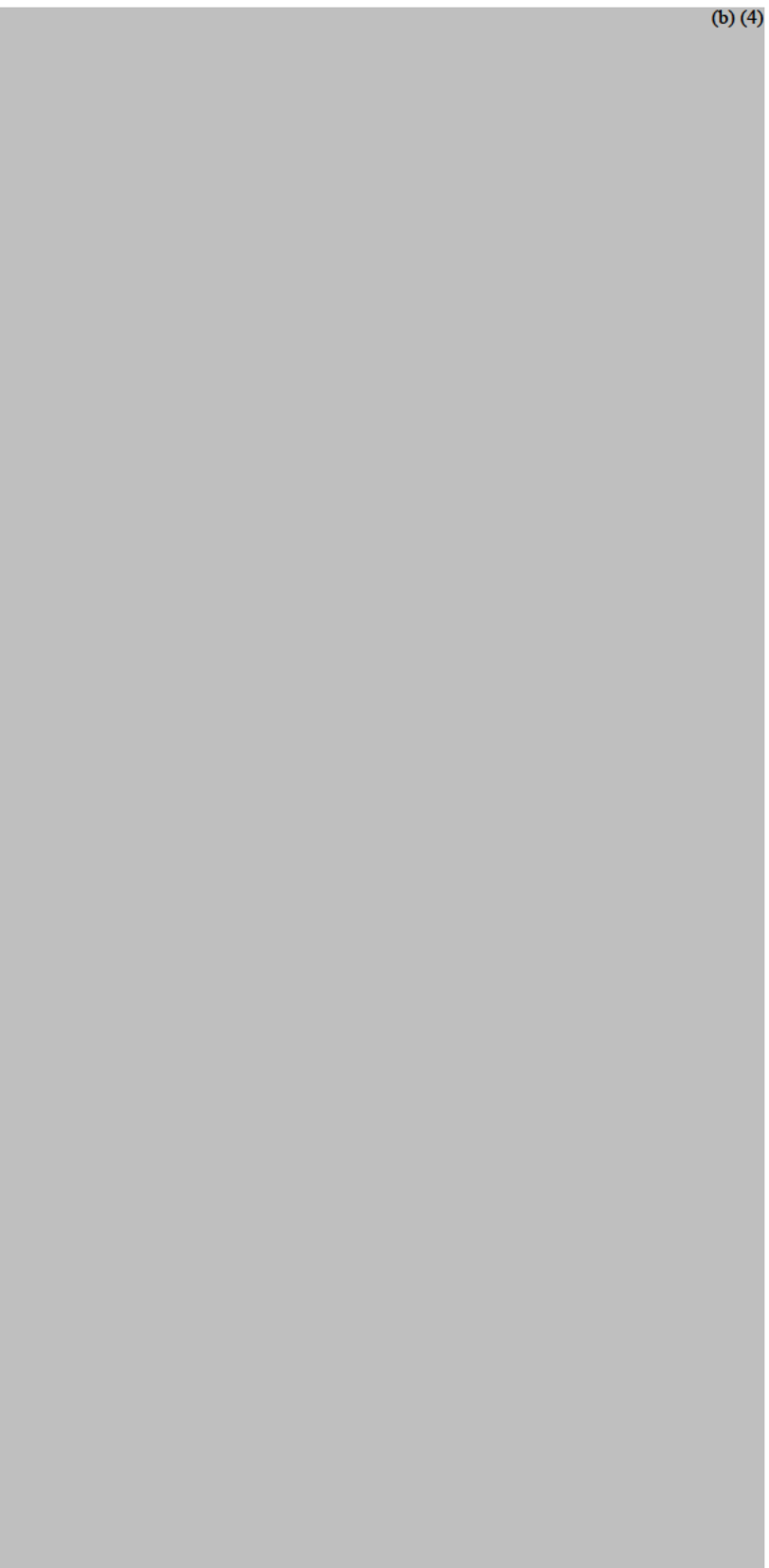
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ASCUS
BIOSCIENCES

Method

Title	**Spore Counting**
Version	**02**
Effective Date	**16Jan2020**
Author	**Rich La**
Approver (Signature & Date)	DocuSigned by: *Martin Mayhew* D1605E1B4C3E49A... **Martin Mayhew** **Vice President - Process Development and Manufacturing** 1/15/2020

Scope

The purpose of this method is to determine the number of viable spores in a sample by counting them in a Petroff-Hausser Haemocytometer.

Safety

Consult the Safety Data Sheet for all reagents prior to handling. Wear appropriate personal protective equipment (safety glasses and gloves) at all times.

Materials

N/A

Equipment

Petroff-Hausser Haemocytometer (Hausser Scientific Cat #3900) with supplied coverslip
Phase Contrast Microscope

Media and Reagents

95% Ethanol
Phosphate-Buffered Saline + 0.05% Tween-20 (PBST)

Component	Amount per 1.0L
(b) (4)	

Method

(b)
(4)

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(4)

(b) (4)

(b) (4)

Reasons for Revision
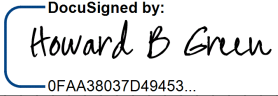
1. Remove organism name from the method title so the method may be used for any spore-forming organisms.

Analysis of *Clostridium beijerinckii* ASCUSDY20 SDP for Heavy Metals & Microbial Contamination

Approvers:

DocuSigned by:

Martin Mayhew

D1005F1B4C3E49A...

12/13/2019

Martin Mayhew
Vice President – Product Development
  & Manufacturing

Date

DocuSigned by:

Patricia A. Williams

5B301285A10643D...

12/12/2019

Patricia A. Williams
Quality

Date

DocuSigned by:

Howard B Green

0FAA38037D49453...

12/12/2019

Howard B. Green
Regulatory

Date

Prepared by
Ascus Biosciences
San Diego, CA

December 2019

Analysis of *Clostridium beijerinckii* ASCUSDY20 SDP for Heavy Metals & Microbial Contamination

Three lots of *C. beijerinckii* ASCUSDY20 SDP were sent for heavy metal and microbial contamination analysis at ⬚⬚⬚⬚⬚ (b) (4) ⬚⬚⬚⬚⬚ (Note *C. beijerinckii* ASCUSDY20 SDP is listed on certificate of analysis as Dairy-20 which was internal name used by ASCUS.)

The ICP-MS/AOAC 2015.01 method was used for the heavy metal analysis of the samples and results are summarized in the following table.

Table 1.   Heavy Metal Analysis of Three Lots of *Clostridium beijerinckii* ASCUSDY20 SDP

Lot Number	Arsenic, ppm	Cadmium, ppm	Lead, ppm	Mercury, ppm
Detection Limit	0.002	0.002	0.002	0.002
18-0202-041-P22	0.227	0.009	0.060	0.004
18-0202-001-P79	0.242	0.084	0.005	0.004
18-0202-001-P73	0.321	0.394	0.051	0.046

ND – None Detected

The methods used for analysis were FDA BAM for Coliforms/E. coli, AOAC 2013.01 for Salmonella and AOAC 2013.10 for Listeria. Results are summarized in the following table.

Table 2.   Microbial Contamination Testing for *Clostridium beijerinckii* ASCUSDY20 SDP

Lot Number	Coliform, CFU/g	E. coli, CFU/g	Salmonella, per 25g	Listeria, per 25g
Requirement	<10	<10	Negative	Negative
18-0202-041-P22	<10	<10	Negative	Negative
18-0202-001-P79	<10	<10	Negative	Negative
18-0202-001-P73	<10	<10	Negative	Negative

C. beijerinckii ASCUSDY20 SDP is intended to be fed as part of the product mixed in a grain premix then further diluted in a total mixed ration or grain supplement. Given the low inclusion rate in the grain mix (5 g/cow/day) and further dilution in the total mixed ration, no heavy metal specification is needed. However, all lots will be tested for microbial contamination at the end of the production of *C. beijerinckii* ASCUSDY20 SDP.

Clostridium beijerinckii **ASCUSDY20 SDP**
Analysis for Heavy Metals & Microbial Contamination

Attachment 1. Certificate of Analysis – Heavy Metal Analysis

(b) (4)

Certificate of Analysis

February 14, 2019

ASCUS BIOSCIENCES
6450 Lusk Blvd, Suite E209
San Diego, CA 92121

(b) (4)

Sample information

Product	Five Dairy - 20 Spray dried microbial powder samples. Store at RT. 1.Lot No. 18-0202-041-P22 2. Lot No. 120318-2 3. Lot No. 120318-3 4. Lot No. 120318-4 5. Lot No. 120318-5
Sampling	Received from Client.
Received	December 19, 2018.

Analytical Results

Analysis Methods	Heavy Metals ICP-MS
Analysis Date	February 12, 2019 to February 14, 2019
Analyst	(b) (4)

Findings

Sample ID	Arsenic, ppm	Cadmium, ppm	Lead, ppm	Mercury, ppm
1. Lot No. 18-0202-041-P22	0.227	0.009	0.060	0.004
2. Lot No. 120318-2	0.252	0.010	0.057	0.002
3. Lot No. 120318-3	0.251	0.009	0.059	0.002
4. Lot No. 120318-4	0.254	0.010	0.057	None Detected
5. Lot No. 120318-5	0.258	0.012	0.061	0.002
Detection Limit, ppm	0.002	0.002	0.002	0.002

Reported by

(b) (4)

(b) (4)

Senior Chemist   Senior Chemist

If there are any questions with this report, please contact "iso@anresco.com".   021419_1   page 1 of 1

(b) (4)

Attachment 2. Certificate of Analysis – Heavy Metal Analysis

(b) (4)

(b) (4)

Certificate of Analysis

January 22, 2019

(b) (4)

ASCUS BIOSCIENCES
6450 Lusk Blvd, Suite E209
San Diego, CA 92121

Sample information

Product	Dairy-20 Spray dried microbial powder. Store at RT. Lot No. 18-0202-001-P79
Sampling	Received from Client.
Received	January 07, 2019.

Analytical Results

Analysis	General Heavy Metal Scan
Methods	Various Methods
Analysis Date	January 07, 2019 to January 22, 2019
Analyst	(b) (4)

Findings

Analysis	Result	Method
Arsenic, ppm	0.242	ICP-MS
Cadmium, ppm	0.084	ICP-MS
Lead, ppm	0.005	ICP-MS
Mercury, ppm	0.004	ICP-MS
pH	4.75	AOAC 981.12
Water Activity	0.076	AOAC 978.18
Moisture, %	3.13	AOAC 927.05
Salt, %	1.00	AOAC 937.09
Water phase salt	24.21	AOAC 937.09, 927.05

Reported by

(b) (4)

(b) (4)

Senior Chemist

Senior Chemist

If there are any questions with this report, please contact "iso@anresco.com".

012219_1   page 1 of 2

(b) (4)

Clostridium beijerinckii ASCUSDY20 SDP
Analysis for Heavy Metals & Microbial Contamination

Attachment 3. Certificate of Analysis –Heavy Metal Analysis

(b) (4)

(b) (4)

Certificate of Analysis

June 17, 2019

ASCUS BIOSCIENCES   (b) (4)
6450 Lusk Blvd, Suite E209
San Diego, CA 92121

Sample information

Product	Dairy-20 Spray Dried Microbial Powder. Store at RT. Lot No. 18-0202-001-P73
Sampling	Received from Client.
Received	June 12, 2019.

Analytical Results

Analysis	General Heavy Metal Scan
Methods	ICP-MS/AOAC 2015.01
Analysis Date	June 12, 2019 to June 17, 2019
Analyst	(b) (4)

Findings

Analysis	Findings (ppm)	Detection Limit (ppm)
Arsenic	0.321	0.003
Cadmium	0.394	0.001
Lead	0.051	0.002
Mercury	0.046	0.001

Reported by
(b) (4)

(b) (4)

Senior Chemist   Senior Chemist

Clostridium beijerinckii ASCUSDY20 SDP
Analysis for Heavy Metals & Microbial Contamination

Attachment 4. Certificate of Analysis – Microbial Contamination Testing (b) (4)

(b) (4)

Certificate of Analysis

December 26, 2018

ASCUS BIOSCIENCES
6450 Lusk Blvd, Suite E209
San Diego, CA 92121

(b) (4)

Sample information

Product	Five Powder Samples: 1. Dairy - 20 Spray dried microbial powder. Store at RT. Lot No. 18-0202-041-P22 2. Dairy - 20 Spray dried microbial powder. Store at RT. Lot No. 120318-2 3. Dairy - 20 Spray dried microbial powder. Store at RT. Lot No. 120318-3 4. Dairy - 20 Spray dried microbial powder. Store at RT. Lot No. 120318-4 5. Dairy - 20 Spray dried microbial powder. Store at RT. Lot No. 120318-5
Sampling	Received from Client
Receive	December 19, 2018

Analytical Results

Methods	FDA BAM - Coliform/E. coli AOAC 2013.01 - Salmonella AOAC 2013.10 - Listeria
Analysis Date	December 19, 2018 to December 26, 2018

Sample	Coliforms cfu/g	E. coli cfu/g	Salmonella per 25g	Listeria per 25g
1. Dairy 20 - Lot No. 18-0202-041-P22	<10	<10	Negative	Negative
2. Dairy 20 - Lot No. 120318-2	<10	<10	Negative	Negative
3. Dairy 20 - Lot No.120318-3	<10	<10	Negative	Negative
4. Dairy 20 - Lot No.120318-4	<10	<10	Negative	Negative
5. Dairy 20 - Lot No. 120318-5	<10	<10	Negative	Negative

Reported by
(b) (4)

(b) (4)

Microbiologist

(b) (4)

Assistant Microbiologist

If there are any questions with this report, please contact "iso@anresco.com".   010819_1   page 1 of 1

(b) (4)

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Attachment 5. Certificate of Analysis – Microbial Contamination Testing

(b) (4)

(b) (4)

Certificate of Analysis

January 21, 2019

ASCUS BIOSCIENCES
6450 Lusk Blvd, Suite E209
San Diego, CA 92121

(b) (4)

Sample information

Product	Dairy-20 Spray Dried Microbial Powder Store at RT. Lot No. 18-0202-001-P79
Sampling Received	Received from Client. January 07, 2019

Analytical Results

Methods	FDA BAM - Coliforms/E. coli AOAC 2013.01 - Salmonella AOAC 2013.10 - Listeria
Analysis Date	January 07, 2019 to January 21, 2019

Analyses	Results
Confirmed Coliforms cfu/g	<10
E. coli cfu/g	<10
Salmonella/25g	Negative
Listeria/25g	Negative

Reported by
(b) (4)

(b) (4)

(b) (4)

Senior Chemist

Assistant Microbiologist

Clostridium beijerinckii **ASCUSDY20 SDP**
Analysis for Heavy Metals & Microbial Contamination

Attachment 6. Certificate of Analysis – Microbial Contamination Testing

(b) (4)

(b) (4)

Certificate of Analysis

July 8, 2019

ASCUS BIOSCIENCES
6450 Lusk Blvd, Suite E209
San Diego, CA 92121

(b) (4)

Sample information

Product   Dairy-20 Spray Dried Microbial Powder. Store at RT. Lot No. 18-0202-001-P73

Sampling   Received from Client.
Received   June 12, 2019

Analytical Results

Methods   FDA BAM - SPC, Coliforms/E. coli
                 AOAC 2013.01 - Salmonella
                 AOAC 2013.10 - Listeria
                 AOAC 2003.07 - Staph

Analysis Date   June 12, 2019 to July 8, 2019

Analyses	Results
Standard Plate Count cfu/g	<100
Coliforms	<10
E. coli	<10
Yeast cfu/g	<10
Mold cfu/g	<10
Salmonella/25g	Negative
Listeria/25g	Negative
CP Staph cfu/g	<10

Reported by

(b) (4)

(b) (4)

SIGNATURE1

Assistant Microbiologist

Suggested Decision Tree for determining the safety of microbial cultures for consumption by humans and animals (Pariza et al, 2015)

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology?
   (If YES, go to 2. If NO, the strain must be characterized and unambiguously identified before proceeding).

2. Has the strain genome been sequenced?
   (If YES, go to 3. If NO, the genome must be sequenced before proceeding to 3.)

3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity?
   (If YES, go to 4. If NO, go to 15.)

4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA?
   (If YES, go to 5. If NO, go to 15.)

5. Does the strain produce antimicrobial substances?
   (If NO, go to 6. If YES, go to 15.)

6. Has the strain been genetically modified using rDNA techniques?
   (If YES, go to 7a or 7b. If NO, go to 8a or 8b.)

7a For strains to be used in human food: Do the expressed product(s) that are encoded by the introduced DNA have a history of safe use in food?
   (If YES, go to 8a. If NO, the expressed product(s) must be shown to be safe before proceeding to 8a.)

7b For strains to be used in animal feed: Do the expressed product(s) that are encoded by the introduced DNA have a history of safe use in feed for the target animal species?
   (If YES, go to 8b. If NO, the expressed product(s) must be shown to be safe for the target animal species before proceeding to 8b.)

8a For strains to be used in human food: Was the strain isolated from a food that has a history of safe consumption for which the species, to which the strain belongs, is a substantial and characterizing component (not simply an 'incidental isolate')?
   (If YES, go to 9a. If NO, go to 13a.)

8b For strains to be used in animal feeds: Was the strain isolated from a feed (for example, silage) that has a history of safe consumption by target animals, for which the species, to which the strain belongs, is a substantial and characterizing component (not simply an 'incidental isolate')?
   (If YES, go to 9b. If NO, go to 13b.)

9a For strains to be used in human food: Has the species, to which the strain belongs, undergone a comprehensive peer-reviewed safety evaluation and been affirmed to be safe for food use by an authoritative group of qualified scientific experts?
   (If YES, go to 10a. If NO, go to 13a.)

9b For strains to be used in animal feeds: Has the species, to which the strain belongs, undergone a comprehensive peer-reviewed safety evaluation and been affirmed to be safe for feed use by an authoritative group of qualified scientific experts?
   (If YES, go to 10b. If NO, go to 13b.)

10a For strains to be used in human food: Do scientific findings published since completion of the comprehensive peer-reviewed safety evaluation cited in question 9a continue to support the conclusion that the species, to which the strain belongs, is safe for use in food?
   (If YES, go to 11a. If NO, go to 13a.)

10b For strains to be used in animal feeds: Do scientific findings published since completion of the comprehensive peer-reviewed safety evaluation cited in question 9b continue to support the conclusion that the species, to which the strain belongs, is safe for use in feed?
    (If YES, go to 11b. If NO, go to 13b.)

11a For strains to be used in human food: Will the intended use of the strain expand exposure to the species beyond the group(s) that typically consume the species in "traditional" food(s) in which it is typically found (for example, will a strain that was isolated from a fermented food typically consumed by healthy adults be used in food intended for an 'at risk' group)?
    (If NO, go to 12a. If YES, go to 13a.)

11b For strains to be used in animal feeds: Will the intended use of the strain expand exposure to the species beyond the target animals that typically consume the species in "traditional" feed(s) in which it is typically found (for example, will a strain that was isolated from silage be used in swine feed)?
    (If NO, go to 12b. If YES, go to 13b.)

12a For strains to be used in human food: Will the intended use of the strain expand intake of the species (for example, increasing the number of foods beyond the traditional foods in which the species typically found, or using the strain as a probiotic rather than as a fermented food starter culture, which may significantly increase the single dose and/or chronic exposure)?
    (If NO, go to 14a. If YES, go to 13a.)

12b For strains to be used in animal feeds: Will the intended use of the strain expand intake of the species (for example, increasing the number of feeds beyond the traditional feeds in which the species is typically found, or using the strain as a probiotic rather than as a silage starter culture)?
    (If NO, go to 14b. If YES, go to 13b.)

13a For strains to be used in human food: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies?
    (If yes, go to 15. If no, go to 14a.)

13b For strains to be used in animal feeds: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies?
    (If yes, go to 15. If no, go to 14b.)

14a The strain is deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption.

14b The strain is deemed to be safe for use in the manufacture of feeds, probiotics, and dietary supplements for animal consumption.

15. The strain is NOT APPROPRIATE for human or animal consumption.

Pariza Decision Tree as applied to *Clostridium beijerinckii* ASCUSDY20

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology?

Yes, go to 2.

2. Has the strain genome been sequenced?

Yes, go to 3.

3. Is the strain free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity?

Yes, go to 4.

4. Is the strain genome free of functional transferable antibiotic resistance gene DNA?

Yes, go to 5.

5. Does the strain produce antimicrobial substances?

No, go to 6.

6. Has the strain been genetically modified using rDNA techniques?

No, go to 8b.

8b. For strains to be used in animal feeds: Was the strain isolated from a feed (for example, silage) that has a history of safe consumption by target animals, for which the species, to which the strain belongs, is a substantial and characterizing component (not simply an 'incidental isolate')?

No, go to 13b.

13b For strains to be used in animal feeds: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies?

No, go to 14b.

14b The strain is deemed to be safe for use in the manufacture of feeds, probiotics, and dietary supplements for animal consumption.

Safety is based on (a) natural occurrence and prevalence of *Clostridium beijerinckii* ASCUSDY20 in the rumen of ruminants; and (b) characterization of the strain to indicate absence of any anticipated virulence factors for pathogenicity or anti- microbial resistance of concern.

Search Strategy for Literature Review: *Clostridium beijerinckii*

A literature search was conducted on 1 December 2019 in order to identify potential information related to the safety and utility of *Clostridium beijerinckii* as a direct fed microbial (DFM) strain for cattle. The overall search strategy is described in Table 1. The NCBI database was reviewed as well as published literature to identify all recognized taxonomic classification of the species Strains falling in this species have previously been classified as *Clostridium acetobutylicum* and *Clostridium butylicum*. Moreover, data on the closely related species, *Clostridium butyricum* was also considered relevant. The relevant database was searched using the keyword/search terms listed in Tables 2A, B and C. Pertinent review articles and authoritative body evaluations (EFSA scientific opinions etc.) were also reviewed to ensure the completeness of literature search (see Table 2C). There were many duplicates identified between the different searches on account of the different nomenclature being widely acknowledged in publications, and of there being a number of pertinent reviews on closely related *Clostridia* species. Considering the relatively large body of available information on closely related *Clostridia* species, the objective of the search was to identify a representative body of information on the species. Reviews and a general google scholar search were also used to identify any additional pertinent information not captured by the initial web of science search.

Table 1:	Literature Search and Selection Strategy	
Step 1	Records identified using selected literature databases	Web of Science
	Total records (titles/abstracts) identified through electronic search	
Step 2	Merge search results and exclude duplicates	
Step 3	Screen titles/abstracts and exclude obviously irrelevant records	
Step 4	Review full texts and assess for relevance and eligibility for inclusion	
Step 5	Use authoritative body opinion and reviews to identify pertinent safety information based primarily on the genus and generally for ruminants	

Table 2A:	Topic Specific Search Terms using Species [*Clostridium beijerinckii*]			
Search strategy for safety of species [Safety Search]	Keywords/search terms [Database: Web of Science]	Term 1	*Clostridium beijerinckii*	Merge, exclude duplicates (n=125)
		Term 2	Tox*(n=97) Pathogen* (n=22) Safe*(n=7) Infection (n=8) Disease (n=10) Mortal* (n=0)	
Search strategy for safety *Clostridium beijerinckii* [Target Animal]	Keywords/search terms [Database: Web of Science]	Term 1	*Clostridium beijerinckii*	Merge, exclude duplicates (n=11)
		Term 2	Cattle (n=0) Cow* (n=9) Bovine (n=1) Ruminant* (n=1) Calves (n=0)	
		Term 1	*Clostridium beijerinckii*	

Search strategy for history of use *of Clostridium beijerinckii*	Keywords/search terms [Database: Web of Science]	Term 2	Food* (n=1) Feed* (n=8)	Merge, exclude duplicates (n=8)

Search: Term 1 in combination with one or more of Term 2; Boolean search techniques were applied.

Table 2B:	Topic Specific Search Terms using Species [*Clostridium acetobutylicum*]			
Search strategy for safety of species [Safety Search]	Keywords/search terms [Database: Web of Science]	Term 1	*Clostridium acetobutylicum*	Merge, exclude duplicates (n=290)
		Term 2	Tox*(n=238) Pathogen* (n=45) Safe*(n=9) Infection (n=11) Disease (n=20) Mortal (n=0)	
Search strategy for safety *Clostridium acetobutylicum* [Target Animal]	Keywords/search terms [Database: Web of Science]	Term 1	*Clostridium acetobutylicum*	Merge, exclude duplicates (n=12)
		Term 2	Cattle (n=0) Cow* (n=5) Bovine (n=3) Ruminant* (n=4) Calves (n=0)	
Search strategy for history of use *of Clostridium acetobutylicum.*	Keywords/search terms [Database: Web of Science]	Term 1	*Clostridium acetobutylicum*	Merge, exclude duplicates (n=346)

Search: Term 1 in combination with one or more of Term 2; Boolean search techniques were applied.

Table 2C:	Topic Specific Search Terms using Species [*Clostridium butylicum*]			
Search strategy for safety of species [General Search]	Keywords/search terms [Database: Web of Science]	Term 1	*Clostridium butylicum* (n=48)	General references on species and properties only identified (relevant studies, n=5)

Search: no Term 2 included on the basis of the limited number of references on the species.

Table 2D:	Topic Specific Search Terms using Species [*Clostridium butyricum*]			
Search strategy for safety of species [Safety Search]	Keywords/search terms [Database: Web of Science]	Term 1	*Clostridium butyricum*	Merge, exclude duplicates (n=358)
		Term 2	Toxi*(n=181) Pathogen* (n=97) Safe*(n=27) Infection (n=68) Disease (n=116) Mortal* (n=10)	

Table 2D:	Topic Specific Search Terms using Species [*Clostridium butyricum*]				
Search strategy for safety [Target Animal Search]	Keywords/search terms [Database: Web of Science]	Term 1	*Clostridium butyricum*	Merge, exclude duplicates (n=26)
		Term 2	Cow* (n=16) Cattle (n=9) Ruminant* (n=1) Calves (n=4)	
Search strategy for history of use [History of Use Search]	Keywords/search terms [Database: Web of Science]	Term 1	*Clostridium butyricum*	Merge, exclude duplicates (n=383)
		Term 2	Food* (n=141) Feed* (n=188)	

Search: Term 1 in combination with one or more of Term 2; Boolean search techniques were applied.

Microbiome Safety for *Clostridium beijerinckii* ASCUSDY20

1   Objectives

The objective of this work is to:

1. Elucidate the roles of rumen microbiome in rumen digestive health via literature review.

2. Identify the typical microbial composition of the rumen microbial community of dairy cows using external datasets and peer reviewed manuscripts.

3. Identify examples and methods of rumen microbiome manipulation in peer reviewed manuscripts.

4. Corroborate if daily administration of *Clostridium beijerinckii* ASCUSDY20 increases its abundance beyond abundances typically observed in the rumen using in-house data.

2   Literature Review

The rumen microbiome is crucial for the digestion of feed and supplies necessary nutrients to ruminants (Faichney, 1996; Huws *et al*., 2018). The rumen hosts a diverse group of microorganisms that work closely to degrade plant materials. The fermentation process converts nearly all dietary carbohydrates to volatile fatty acids (VFA), predominantly butyrate, acetate, and propionate. These three major VFAs play key roles in host metabolism. The butyrate pool in rumen is the smallest of the three (Sutton *et al*., 2003). It is predominantly metabolized by rumen mucosa and almost all of the absorbed butyrate was converted to ketone bodies (Weigand *et al*., 1975; Cook *et al*., 1969). Studies have also linked butyrate to the development of rumen papillary and calf gastrointestinal tracts (Weigand *et al*., 1975; Górka *et al*., 2018). Further, direct infusion of butyrate into the rumen has shown increases in milk fat production without changing milk yield (Huhtanen *et al*., 1993). Unlike butyrate, acetate and propionate are both absorbed by rumen and passed to extra-ruminal tissues for metabolism (Cook and Miller, 1965). Propionate, in particular, can be converted into glucose via gluconeogenesis in the liver. Studies show that gluconeogenesis provides up to 90% of the glucose required by ruminants, and over half of the glucose produced is derived from propionate (Leng *et al*., 1967; Young, 1977). Thus, a large rumen propionate pool is needed to support the basic ruminant metabolism. Yost *et al*., (1977) reported that rumen propionate pool size is directly related to the amount of feed intake and significant differences between individuals were observed, highlighting the rumen

fermentation differences among animals. In addition, direct infusion of propionate into the rumen has been shown to increase milk protein production, but decrease milk yield (Rook and Balch, 1961). Acetate absorbed through rumen epithelium was predominantly metabolized by extra-ruminal tissues other than liver (Cook and Miller, 1965). Direct infusion of acetate into the rumen has been shown to improve the yield of milk, as well as the amount of milk fat produced (Rook and Balch, 1961). Interestingly, Sabine and Johnson (1964) found only 40-50% of the infused acetate was used by the host, suggesting acetate may play an equally important role if not more in the development of rumen microbiome. The study also reported a large variability of acetate usage among animals, again highlighting the individual host differences which the rumen microbiomes are likely contributing to.

Besides its importance in fulfilling ruminant carbon needs, rumen microorganisms are also pivotal in providing nitrogen. Published studies estimate that approximately 60-90% of protein absorbed by ruminant duodenum arises from a microbial source (Wallace *et al.*, 1997; Broudiscou and Jouany, 1995). The association between rumen nitrogen use efficiency and microbiome has also been widely reported (Huws *et al.*, 2018; Bach *et al.*, 2005; Edwards *et al.*, 2008). To further elucidate the roles of rumen microbiome, Lin *et al.* (2019) identified microbial activities and their corresponding host genetic responses, emphasizing the symbiotic relationship between host nutrient needs and rumen microorganisms. Therefore, changes in rumen microbiome could directly influence ruminant nutrient balance.

The importance of rumen microbiome, especially its unique ability in cellulose degradation, has long been discussed (Woodman and Stewart, 1928; Woodman, 1930). Hungate (1957) attempted to characterize the rumen microbiome by anaerobic cultivation. These studies provided a glimpse into rumen bacterial diversity as well as the metabolic potential of select bacterial species. However, the development of molecular biology and Next-Generation Sequencing (NGS) techniques have revealed that many of the cultivation techniques leveraged by Hungate only characterized a small proportion of the rumen microbial community. A large proportion of the rumen microbiome is considered "unculturable", and hence dismissed in early rumen microbiology experiments (Jannasch and Jones, 1959; Staley, 1985; Pace, 1997; Steen *et al.*, 2019). Since then, the use of molecular techniques (Pace, 1997; Zuckerkandl and Pauling, 1965; Schwartz and Dayhoff, 1978; Woese *et al.*, 1990) leveraging NGS have greatly advanced our ability to characterize rumen microbiome and its associations with animal health and nutrition, as well as environmental factors (Wallace *et al.*, 1997; Rodriguez-R and Konstantinidis, 2014; Jami and Mizrahi, 2012; Kumar *et al.*, 2015; Wallace *et al.*, 2019; Petri *et al.*, 2013; Huws *et al.*, 2018; Henderson *et al.*, 2015; Deusch *et al.*, 2017; Mizrahi and Jami, 2018; Sasson *et al.*, 2017; Weimer, 2015; Furman *et al.*, 2020).

Marker gene amplicon sequencing is one of the most commonly used methods of rumen microbiome characterization (Sirohi *et al.*, 2012). Typically, the small subunit ribosomal RNA (16S rRNA) gene is used to evaluate bacterial and archaeal community composition, while the internal transcribed spacer (ITS) between the 18S and 28S rRNA is used to characterize fungal community composition (Mizrahi and Jami, 2018). Several studies have linked the rumen microbiome profile to animal performance and milk production and is now considered an indicator of rumen digestive health (Jami and Mizrahi, 2012; Kumar *et al.*, 2015; Lima *et al.*, 2015). Rumen microbiome is highly variable depending on several factors, including age, breed,

diet, location, farm management practices, and lactation stage (Wallace *et al*., 2019; Henderson *et al*., 2015; Furman *et al*., 2020; Pitta *et al*., 2016). To better study the microbiome in context of the observed individuality, many studies have focused on identifying and characterizing the core rumen microbiomes (Jami and Mizrahi, 2012; Kumar *et al*., 2015; Wallace *et al*., 2019; Petri *et al*., 2013; Henderson *et al*., 2015; Furman *et al*., 2020; Lima *et al*., 2015; Xue *et al*., 2018; Kittelmann *et al*., 2013; Fouts *et al*., 2012). The concept of core microbiome, a common assemblage of microorganisms that exists in or is associated with a specific habitat, was first introduced and applied to differentiate human microbiomes associated with healthy and diseased conditions (Turnbaugh *et al*., 2007; Turnbaugh and Gordon 2009; Turnbaugh *et al*., 2009). Since then, core microbiomes have been identified in a broad spectrum of environments including agroecosystems, monogastric animals, and ruminants (Shade and Handelsman, 2012; Yeoh *et al*., 2017; Toju *et al*., 2018; Lowe *et al*., 2012; Dougal *et al*., 2013).

The rumen microbial community composition is constantly in flux. The microbial population has been shown to change over time in response to a variety of factors, including diet composition, time after feeding, season, and stage of lactation. Additionally, there are groups of microorganisms that are unique to particular breeds of cow (i.e. Jersey or Holstein), regions, and individual animals that further increase the inherent complexity of the microbial community native to the rumen. Despite this variability, there is a core microbiome that appears in the majority of animals. This core has been investigated at Ascus Biosciences, as well as in independent academic studies. Although the results are variable at times and defining a "normal healthy" rumen is challenging, there are several phyla that tend to appear across all ruminants. Henderson *et al*. (2015) reported 32 different species of ruminants globally shared a core assembly of rumen bacteria. Consistent with other studies (Jami and Mizrahi, 2012; Deusch *et al*., 2017; Lima *et al*., 2015; Xue *et al*., 2018; Jami *et al*., 2014; Schären *et al*., 2018), members of Bacteroidetes, Firmicutes, Proteobacteria, and Fibrobacteres were among the topmost abundant bacteria identified regardless of animal origin and diet. The fungal rumen community, although much less abundant than the bacterial rumen community, tends to fall into the following phyla: Ascomycota, Basidiomycota, Neocallimastigomycota, and Zygomycota (Kumar *et al*., 2015; Lima *et al*., 2015; Kittelmann *et al*., 2013; Fouts *et al*., 2012; Tapio *et al*., 2017; Langda *et al*., 2020; Dias *et al*., 2017; Paul *et al*., 2018; Belanche *et al*., 2019; Mendes de Almeida *et al*., 2012; Vargas-Bello-Pérez *et al*., 2016; Ishaq *et al*., 2017). Neocallimastigales used to be an order within Chyrtridiomycota, however in 2012, these anaerobic fungi were placed into a separate phylum called Neocallimastigomycota (Adl *et al*., 2012). Although this change was proposed 7 years ago, some species of Neocallimastigomycota are still listed as members of Chyrtridiomycota in public databases. For the sake of clarity, instances of 'Chytridiomycota' have been replaced with 'Neocallimastigomycota' in this report.

Many published manuscripts described the rumen bacterial dynamics. Studies reporting the core bacterial communities from dairy rumen (Jami and Mizrahi, 2012; Wallace *et al*., 2019; Petri *et al*., 2013; Furman *et al*., 2020; Lima *et al*., 2015; Xue *et al*., 2018; Dias *et al*., 2017) and a wide range of ruminants (Henderson *et al*., 2015) are summarized in Table 1. Ascus has also conducted surveys and the results corroborate published numbers (Table 2).

Table 1. The Average Abundance of Major Rumen Bacterial Phyla from Published Studies.

Major Rumen Bacterial Phylum	Percent Relative Abundance								
	Bacterial Core Microbiome								
	Adult Dairy Cows						Pre-weaning Dairy Calves		Ruminants (32 species)
	Xue et al., 2018	Petri et al., 2013	Jami et al., 2012	Lima et al., 2014[a]	Wallace et al., 2019[b]	Furman et al., 2020[c]	Dias et al., 2017[d]	Furman et al., 2020[e]	Henderson et al., 2015[f]
Bacteroidetes	20.68±0.18	32.8	51	33.6-40.7	56	1-75	15-30	1-75	38.7±1.4
Fibrobacteres	0.86±0.02	0.1-15	0.02-0.48	<1	6	<1	NA	NA	3.1±0.1
Firmicutes	21.67±0.18	43.2	41.6	42.5-49.65	16	10-80	30-90	10-80	44.2±1.8
Proteobacteria	0.52±0.01	14.3	5.46	1-12	8	1-70	1-10	1-70	2.8±0.1
Tenericutes	0.44±0.01	NA	0.69	1-3	<1	<1	NA	NA	1.4±0
Spirochaetes	1.35±0.04	0.5-15	<1	<1	5	1-5	NA	NA	1±0

[a] values were estimated from Fig 1

[b] values estimated from Fig 1B

[c] values estimated from Fig 2A (60 - 700 days of life)

[d] pre-weaning calf (7-63 days old) rumen microbiome. Values estimated from Figure 2B

[e] values estimated from Fig 2A (1 - 59 days of life)

[f] approximation from supplementary Table 1 using the most abundant groups

Table 2.   **The Average Abundance of Major Rumen Bacterial Phyla from Ascus Surveys.**

Major Rumen Bacterial Phylum	Percent Relative Abundance			
	Ascus Conducted Surveys			
	Adult Diary Cows			
	Survey 1	Survey 2	Survey 3	Survey 4
Bacteroidetes	36.67	24.75	36.339	44.35
Fibrobacteres	1.53	3.71	0.49	1.15
Firmicutes	46.82	61.85	48.41	46.98
Proteobacteria	5.49	3.63	11.2	3.36
Tenericutes	1.26	1.2	0.43	0.7
Spirochaetes	2.72	1.7	0.66	0.55

Despite the recognition of their significant roles in rumen, the diversity characterization of rumen fungal communities is lagging far behind rumen bacteria (Mizrahi and Jami, 2018; Comtet-Marre *et al.*, 2017). This is due to: 1) the understanding of fungi is generally limited to date and frequently, the fungal community profiles were not reported; 2) fungal marker genes varied largely among fungal phylogeny and researches frequently target different regions that apply to their specific research questions. For example, published manuscripts, Kittleman, *et al.*, (2013), Dias, *et al.* (2017), Paul *et al.* (2018), and Tapio *et al.* (2017), describing the dairy rumen fungal community using an ITS primer set (MN100 and MNGM2) bias towards members of Neocallimastigomycota. This led to the primary identification of Neocallimastigomycota in dairy rumen and neglecting other fungal groups. Below, from the available and applicable literature, we summarized the average abundance of major fungal groups in dairy rumen (Kumar *et al.*, 2015; Fouts *et al.*, 2012; Mendes de Almeida *et al.*, 2012; Ishaq *et al.*, 2017) and other ruminants (Langda *et al.*, 2020; Belanche *et al.*, 2019) (Table 3). Ascus conducted survey results are reported in Table 4. The average abundance of major rumen fungal phyla from Ascus surveys are also consistent with the published studies.

Table 3. **The Average Abundance of Major Rumen Fungal Phyla from Published Studies.**

Major Rumen Fungal Phylum	Percent Relative Abundance					
	Dairy Cow				Other Ruminants	
	Kumar et al., 2015	Mendes de Almeida et al., 2012[a]	Ishaq et al., 2017[b]	Fouts et al., 2012[c]	Belanche et al., 2019[d]	Langda et al., 2019[e]
Ascomycota	27	85	5-68	47-68	1-9	18-30
Basidiomycota	3	Cannot be cultivated aerobically	1-3	2-10	8-20	< 1
Neocallimastigomycota	1		26-92	30-50	71-92	52-78
Zygomycota	< 1	15	< 1		NA	< 1
unidentified	68	NA	1-5	1-5	NA	0.1-0.5

a aerobic cultivation based
b values estimated from Fig 2
c values estimated from Fig 2C
d values estimated from Fig 4B
e values estimated from Fig 2D

Table 4.   **The Average Abundance of Major Rumen Fungal Phyla from Ascus Conducted Surveys.**

Major Rumen Fungal Phylum	Percent Relative Abundance	
	Ascus Surveys (Dairy Cows)	
	Survey 1	**Survey 2**
Ascomycota	36.57	58.09
Basidiomycota	12.54	0.042
Neocallimastigomycota	50.86	41.86
Zygomycota	0.0047	0.0003
unidentified	0.03	0

As more rumen microbiomes were studied, it became clear that diet was the major determinant of observed microbiome differences (Kumar *et al*., 2015; Deusch *et al*., 2017; Mizrahi and Jami, 2018; Belanche *et al*., 2019; Johnson and Johnson, 1995; Brulc *et al*., 2009; Carberry *et al*., 2014). This indicates the direct impact of diet on rumen microbial populations. Indeed, few strong co-occurrence patterns were observed among rumen microbes, suggesting that shifts within core microbiome were based on the pool of available metabolites produced during ingesta fermentation. Hence, modifying either diet or microbiome could influence the rumen fermentation process (Wallace *et al*., 2019; Furman *et al*., 2020; Moraïs and Mizrahi, 2019; Belanche *et al*., 2012).

Numerous studies suggested that microbiome shifts improved digestibility (Wallace *et al*., 2019; Weimer, 2015; Comtet-Marre *et al*., 2017; Moraïs and Mizrahi, 2019; Yáñez-Ruiz *et al*., 2015). Based on the current literature, Moraïs and Mizrahi (2019) summarized that multiple microbial community states exist within the rumen depending on the rumen metabolic needs. The flow of metabolites and energy were passed on from one functional group to the next rather than from one group to another. While individual microbial species may be able to carry out similar functions, Moraïs and Mizrahi (2019) hypothesize that microbial interactions drive larger changes in overall fermentation patterns. Hence, identifying the optimal microbial interactions could improve digestibility (Weimer, 2015). Sasson *et al*. (2017) reported that the differences in cows' ability to harvest energy was correlated with a group of heritable rumen microorganisms. Wallace *et al*. (2019) extended the study with a bigger cohort of animals. Similar results were reported, where specifically that rumen digestibility differences were associated with heritable core rumen microbiomes. This is also consistent with other studies showing that early colonization of microbes through vaginal birth could improve rumen digestibility significantly (Furman *et al*., 2020; Yáñez-Ruiz *et al*., 2015). While a microbiome-led breeding program could be used to preserve the optimal microbial interactions and improve rumen digestibility, it is not the most efficient and the outcome may be difficult to predict. Many other methods have been reported to promote efficient microbial interactions by shifting rumen microbiome (Weimer, 2015).

3   Altering the Microbiome

Throughout the history of agriculture, humans have long been manipulating rumen microbiomes to enhance rumen digestibility and fermentation profiles. For centuries, Swedish farmers have fed cud from healthy cattle to another with ruminal indigestion (Brag and Hansen, 1994). This method was later scientifically evaluated and became a common practice called rumen transfaunation (Brag and Hansen, 1994; DePeters and George, 2014). Ribeiro *et al.* (2017) recently conducted a study where 70% of the barley fed domestic cattle's rumen content was replaced by foraging bison rumen content repeatedly. The study found the procedure significantly improved cattle N digestibility. In another study, mixed rumen contents from two healthy cows were fed to 45 cattle with primary and secondary digestive issues (Steiner *et al.*, 2020). After the transfaunation, it was observed that the sick animals had increased appetite and improved rumen digestibility. However, the exotic microbiome may not consistently establish due to significant host physiological differences. While the introduced microbiome did not interfere with normal rumen function, inconsistent establishment of a new microbiome was observed, and some were reverted back to a state similar to the original microbiome (Zhou *et al.*, 2018; Weimer *et al.*, 2010).

Alternative to transfaunation, in-feed supplementation of native and non-native microorganisms have also been used to treat rumen indigestion (McAllister *et al.*, 2011; Nagpal *et al.*, 2015). Unlike transfaunation, the process promotes the shifts of the native rumen microbiome instead of introducing exotic microbial communities. In-feed supplementation is non-invasive and eliminates the danger of accidental pathogen feeding. Many different microorganisms have been isolated and used as direct fed microbial products (DFM) in treating rumen digestion issues (McAllister *et al.*, 2011; Nagpal *et al.*, 2015; Meissner *et al.*, 2010; Stein *et al.*, 2006). The DFMs in use today include members of bacteria and fungi. Studies have shown that they are capable of out-competing rumen pathogens, moderating rumen pH (by utilizing overproduced lactic acid or increasing the production of volatile fatty acids propionate) and improving fiber digestion by excreting cellulolytic/hydrolytic enzymes. Thus, introducing microorganisms to promote microbiome changes and to optimize microbial interactions is a valid method of improving rumen digestibility.

To compare the impact of DFM and diet on rumen microbiome, Ishaq *et al.* (2017) conducted a study where yeast was administered to animals fed either a high-fiber diet or a high-grain diet and the changes in rumen fungal and protozoal microbiomes were evaluated. This experiment showed that diet had far greater influence on the composition of the microbiome than the supplementation of yeast. In Table 2 from the manuscript (Table 5 below), the AMOVA analysis shows that feeding of yeast created no significant difference in fungal microbiome composition between control and treatment cows on the same diet type (e.g. high-fiber yeast vs. high-fiber control). Similar results were observed for ANOSIM analysis. Diet, however, did create statistically significant differences in microbiome composition.

Conklin (2018) conducted a similar study involving Bovamine, a commercially available bacterial DFM product of Chr. Hansen (Hørsholm, Denmark) that consists of *Lactobacillus acidophilus* LA51 and *Propionibacterium freudenreichii* NP24. In the study, Bovamine was administered daily to dairy cows fed with a low starch diet for the first 5 weeks of the trial.

Animals were abruptly switched to a high starch diet the 6th week of the trial. The study found that the effects of neither ration starch content or Bovamine treatment were significant, but there was a trend of microbiome separation by starch content (Figure 1). There was also a trending difference in the most abundant bacterial families between the two diet types, however, the differences induced by the DFM was minimal or none (Figure 2). Thus, although DFM supplementation may impact the rumen microbiome and fermentation, the amount of change is not as dramatic and significant as diet formulation.

Although diet contributes to the most changes observed in the rumen microbiome, introducing DFM under the same diet can induce beneficial shifts in microbial populations within natural ranges. Westergaard (2015) fed *Bacillus pumilus* DFM to dairy cows. The study reported an insignificant increase in Firmicutes from 14.1% to 15.8% and a decrease of Bacteroidetes from 64.1% to 62.3% in rumen fluid of animals received the DFM. Its companion study reported that the animals receiving the DFM were more efficient at feed conversion than the control animals, although not significantly (Luan *et al.,* 2015).

A study conducted by Le and colleagues (2017) reported that dairy calves administered DFM *Bacillus amyloliquefaciens* gained 20% more weight and suffered less diarrhea than the control group. Interestingly, its companion study observed supplementing DFM *B. amyloliquefaciens* did not change dairy calf rumen microbiomes significantly, despite that colonization of the DFM strain was confirmed in rumen (Schofield *et al.,* 2018). The study did notice periodic changes in bacterial populations, such as members of *Prevotella*, Phascolarctobacterium, and Succinivibrionaceae.

Fomenky and colleagues (2018) also compared the rumen digesta microbiome of pre- and post-weaned calves fed with control diet alone and control diet supplemented with DFM *Saccharomyces cerevisiae boulardii* (SCB) or *Lactobacillus acidophilus* (LA). The study found that supplementing DFMs did not significantly change the overall rumen microbial community structures, where the p-values for alpha diversity indices ranged from 0.051 to 0.992 and the p-value for beta diversity (PERMANOVA) was 0.512. However, genus group *Ruminococcaceae_UCG-008*, a member of Firmicutes, was 9 fold less abundant in post-weaned rumen supplemented with SCB than the control. No significant changes at the genus level in rumen of animals administered LA were reported. The study also predicted that pathways involved in lipid and protein metabolism and cellular processes were more abundant in pre-weaned rumen administered DFMs. Once weaned, no predicted pathways in rumen digesta were significantly different between control and LA fed animals and riboflavin metabolism was the only significantly more abundant pathway in SCB fed animal rumen digesta than control.

These studies demonstrated that DFMs could induce minor shifts within rumen microbiome without significantly changing microbial community structures. Further, these minor changes within rumen microbiome could promote better microbial interactions and improve the overall rumen feed digestibility.

Table 5. Table 2 from Ishaq *et al.*, 2017. Comparison of treatments by AMOVA, ANOSIM, and UniFrac, for rumen fungi and protozoa for cows receiving two dietary treatments with or without yeast supplementation under SARA conditions.

| | Fungal ITS | | | | | Protozoal 18S | | | | |
| | AMOVA | ANOSIM | | Weighted UniFrac | | AMOVA | ANOSIM | | Weighted UniFrac | |
	P	R	P	W	P	P	R	P	W	P
Locat on	**	0.13	*	0.65	**	*	0.08	**	0.87	**
Ep mura x F ud	**	0.05	ns	0.65	**	*	0.10	*	0.99	**
Ep mura x So d	Ta	0.06	ns	0.55	**	*	0.08	*	1	**
F ud x So d	**	0.28	**	0.77	*	*	0.07	*	0.61	**
HF x HG	ns	0.93	**	1	**	**	0.10	**	0.65	**
C x Y	**	0.01	ns	0.48	**	Ns	0.00	Ns	0.61	**
Treatment	**	0.51	**	0.83	*	**	0.15	**	0.87	**
HFC x HGC										
Ep mura	**	0.91	**	1	**	*	0.40	*	1	**
F ud	n/a	n/a	n/a	n/a	n/a	ns	0.00	ns	0.65	**
So d	**	0.95	**	1	**	ns	0.11	ns	0.74	**
HFY x HGY										
Ep mura	**	0.82	**	1	**	ns	0.31	*	1	Ns
F ud	n/a	n/a	n/a	n/a	n/a	ns	0.19	*	0.5	**
So d	Ta	0.85	T1	1	**	ns	0.00	ns	0.85	**
HFC x HFY										
Ep mura	ns	0.03	ns	0.61	*	ns	−1.8	ns	0.96	**
F ud	ns	0.01	ns	0.55	*	ns	0.03	ns	0.66	**
So d	ns	0.00	ns	0.79	*	ns	0.00	ns	0.65	**
HGC x HGY										
Ep mura	ns	0.02	ns	0.74	**	ns	0.31	*	0.95	*
F ud	n/a	n/a	n/a	n/a	n/a	ns	0.00	ns	0.72	**
So d	ns	0.00	ns	0.63	**	ns	0.02	ns	0.67	**
HFC x HGY										
Ep mura	**	0.84	**	1	**	*	0.32	Ta	0.95	*
F ud	n/a	n/a	n/a	n/a	n/a	ns	0.00	ns	0.53	**
So d	**	0.84	**	1	**	ns	0.00	ns	0.74	**

aValues were significant only before Bonferroni correction.

Diets include high fiber (HF) or high grain (HG), Locations include Epimural (E), fluid (F), or solid (S), and treatments include yeast (Y) or Control (C). Significance is determined as *P* < 0.05,

P < 0.001, **P* > 0.05 (ns), or not enough comparisons to make (n/a). Significance was adjusted by Bonferroni where appropriate.

Figure 1.   **Multidimensional scaling (MDS) of rumen fluid samples collected during the low starch and the high starch ration by treatment (BOV vs. CON).**

Note: For figure simplicity, the low 23.8% starch ration is represented as "20", and the high 31.1% starch ration is represented as "30".
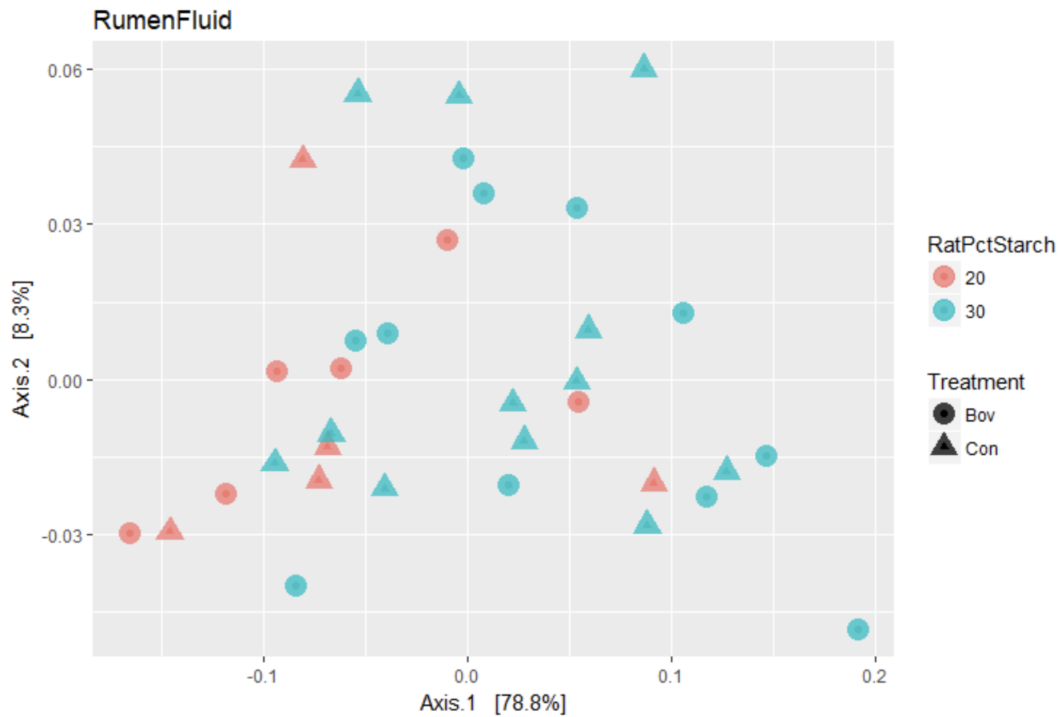
Note: Figure 17 of Conklin (2018) study (Experiment 2).

Figure 2. **Heatmap of bacteria families identified in rumen fluid samples collected during the low starch and the high starch ration by treatment (BOV vs. CON).**

Note: For figure simplicity, the low 23.8% starch ration is represented as "20", and the high 31.1% starch ration is represented as "30".
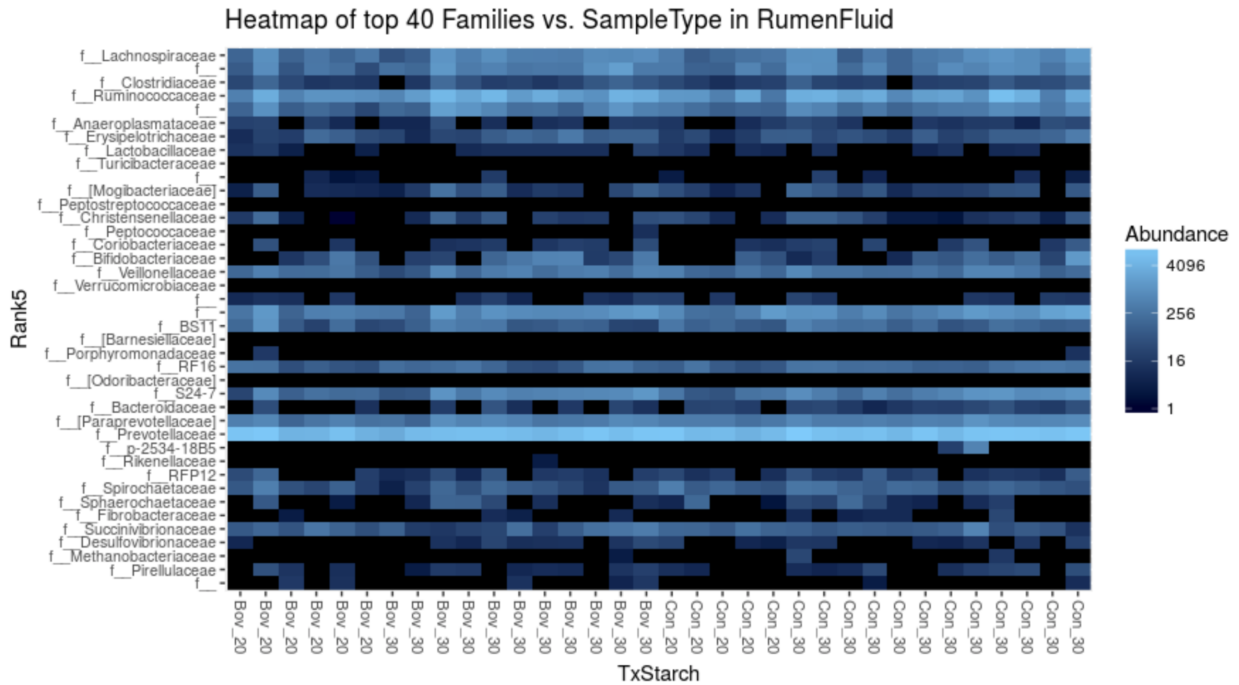
Note: Figure 16 of Conklin (2018) study (Experiment 2).

4 Typical microbiome composition of dairy cows receiving *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21

Ascus conducted an experiment to assess the effects of the administration of native rumen microbes on the rumen microbiome community. The experiment was conducted on 24 dairy cows (8 animals per group): one group of animals received *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21 ("Microbes 1"), a second group received *C. beijerinckii* ASCUSDY20, *P. kudriavzevii* ASCUSDY21, and another native rumen bacterium ("Microbes 2"), and the third group served as control ("No microbes"). The average abundance of major fungal phyla and major bacterial phyla were reported in Table 6 and Table 7, respectively. For the ease of comparison, the abundance of major rumen fungal and bacteria phyla from published literature were also included. In this administration experiment, it can be seen that the addition of *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21 to dairy cows did not significantly alter the rumen fungal or bacterial composition when compared to the control group. Abundances of all fungal and bacterial phyla are within the standard ranges observed in animals not fed native rumen microbes. The average abundance of each phylum tended to be similar across experimental groups. The abundance of all fungal and bacterial phyla is also within the ranges reported in literature (Table 6 and Table 7). Therefore, directly feeding *C. beijerinckii* ASCUSDY20 and P. kudriavzevii ASCUSDY21 did not alter dairy rumen bacterial communities beyond their natural states. This corroborates with Ascus' assessment that administering *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21 to dairy cows do not shift their rumen microbiomes beyond the natural ranges.

Table 6. Abundance of Major Rumen Fungal Phyla from the Ascus Experiments as Compared to Published Data.

Major Rumen Fungal Phylum	Percent Relative Abundance						
	Ascus Experiment			Published Dairy Rumen Data			
	Microbes 1	Microbes 2	No microbes	Kumar et al., 2015	Mendes de Almeida et al., 2012[a]	Ishaq et al., 2017[b]	Fouts et al., 2012[c]
Ascomycota	31.89	31.33	31.5	27	85	5-68	47-68
Basidiomycota	7.33	7.99	9.63	3	15	1-3	2-10
Neocallimastigomycota	60.42	60.16	58.06	1	Cannot be cultivated aerobically	26-92	30-50
Zygomycota	0.00091	0.0003	0.0016	<1		<1	
unidentified	0.46	0.52	0.8	68	NA	1-5	1-5

[a] aerobic cultivation based
[b] values estimated from Fig 2
[c] values estimated from Fig 2C

Table 7. Abundance of Major Rumen Bacterial Phyla from the Ascus Experiment as Compared to the Published Data.

Major Rumen Bacterial Phylum	Percent Relative Abundance								
	Ascus Experiment			Published Dairy Rumen Data					
	Microbes 1	Microbes 2	No microbes	Xue et al., 2018	Petri et al., 2013	Jami et al., 2012	Lima et al., 2014[a]	Wallace et al., 2019[b]	Furman et al., 2020[c]
Bacteroidetes	35.53	36.02	36.3	20.68±0.18	32.8	51	33.6-40.7	56	1-75
Fibrobacteres	0.43	0.42	0.54	0.86±0.02	0.1-15	0.02-0.48	<1	6	<1
Firmicutes	55.73	54.87	54.56	21.67±0.18	43.2	41.6	42.5-49.65	16	10-80
Proteobacteria	4.45	4.47	4.66	0.52±0.01	14.3	5.46	1-12	8	1-70
Spirochaetes	0.97	0.72	0.57	0.44±0.01	NA	0.69	0.69	<1	<1
Tenericutes	0.53	0.69	0.65	1.35±0.04	0.5-15	<1	<1	5	1-5

[a] values were estimated from Fig 1
[b] values estimated from Fig 1B
[c] values estimated from Fig 2A (60 - 700 days of life)

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FINAL REPORT

PREPARED BY / DATE: ⬛ (b) (4) / May 25, 2016

PROJECT NUMBER: DE1601

STUDY TITLE: Rumen microbial inoculation efficacy trial.

INVESTIGATOR: ⬛ (b) (4)

BIOSTATISTICIAN: ⬛ (b) (4)

MONITORS: ⬛ (b) (4)

SPONSOR: Ascus Biosciences, Inc.

OBJECTIVE: Evaluate the effect of inoculating an Ascus Biosciences selection of rumen microbes on milk composition and yield

DATE OF INITIATION: January 18th, 2016

DATE ENDED: March 9th, 2016

(b) (4)

Signature: ⬛ (b) (4)   **Date:** May 25 h, 2016

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EXECUTIVE SUMMARY

A total of 16 multiparous Holsteins cows were brought into ⬛⬛⬛⬛ (b) (4) facilities and individually housed for a total of 52 days. All cows underwent a 10-day period for surgery recovery and adaptation to new facilities and diet. Cows were randomly allocated to two study groups; a) Inoculated: A selection of microbes suspended in buffer solution were inoculated via ruminal cannula once a day during the intervention period; and, b) Control: Cows were inoculated only with buffer control. The intervention period lasted a total of 32 days. Also, outcomes of interest were measured for an additional 10 days after the last inoculation day. A treatment by week interaction was observed for milk yield, fat corrected milk (FCM), energy corrected milk (ECM), and protein yield. A tendency for a treatment by week interaction was also observed for fat yield, feed efficiency (FE), and rumen pH. The interaction for yields was mainly the result of milk yield diverging between the two treatments within the first 2-3 weeks of the study and coming back together toward the end of the Intervention period. A tendency for a higher milk fat percentage for Inoculated vs. the Control was observed. Although the treatment by week interaction was not significant, it can be observed that milk fat percentages was numerically similar within the first two weeks due probably to adaptation and numerically higher for Intervention during weeks three to five. The difference on milk fat percentage was not observed during the follow-up period when cows were not inoculated with microbes. The results obtained in this study are very promising and encourage to further research efficacy of these or additional microbes on milk yield and composition with a larger number of animals.

JUSTIFICATION AND HYPOTHESIS

Ascus Biosciences identified rumen microbial populations which are affected by diet-induced changes in milk fat composition. Therefore, the hypothesis was that inoculating these microbes directly into the rumen would increase milk fat content.

OBJECTIVE

The study objective was to evaluate the effect of inoculating an Ascus Biosciences selection of microbes on milk composition and yield.

MATERIALS AND METHODS

Animals and Facilities

A total of 16 cows were enrolled into the study. The cows were multiparous Holsteins (second and third lactation) that were brought on January 18, 2016 from a local dairy farm into ⬛⬛⬛⬛ (b) (4) facilities (⬛⬛ (b) (4) ⬛ (b) (4)). The animal selection criteria included cows between 60 and 120 days in milk (DIM), daily milk production of 36 kg or more, and somatic cell count (SCC) below 200,000 cells/mL in accordance with the previous DHIA monthly test.

Upon arrival, cows were housed individually in box stalls bedded with almond shells where they were fed twice a day total mixed ration (TMR) diet offered at libitum and had free access to water except for short periods during milking. Cows were milked twice a day (4:30 am and 4:00 pm) at a conventional milking parlor. In the two days after arrival, all cows were surgically fitted with a ruminal cannula on the left flank fossa (Bar Diamond 10 cm 1 C Cannula, Parma, ID).

Experimental Design

Treatment Groups

The cows were randomly allocated to two study groups of 8 cows each:

Inoculated: A selection of microbes suspended in buffer solution personnel were inoculated via ruminal cannula once a day during the intervention period. Cows assigned to I received study IDs 1, 3, 5, 7, 9, 11, 13 and 15.

Control: Cows were inoculated only with buffer control once a day during the intervention period. Cows assigned to C received study IDs 2, 4, 6, 8, 10, 12, 14 and 16.

Study Periods

Pre-Intervention Period

All cows underwent a 10-day period for surgery recovery and adaptation to new facilities and diet. During this period, _____ (b) (4) personnel conducted daily health assessments.

Intervention Period

Immediately after the morning milking cows were inoculated via the rumen cannula by Ascus Biosciences personnel for 32 days.

Post-Intervention Period

Outcomes of interest were measured for an additional 10 days after the last inoculation day.

Rumen Inoculation

Each animal was either inoculated with microbes or with a buffer control via the ruminal cavity in accordance to Ascus Biosciences protocol.

Sampling and Measurements

Feed Intake

Animals were fed twice a day individually in separate feed containers after the morning and afternoon milkings. Feed weights were recorded twice a day at each feeding during Pre-Intervention days 5 to 10, Intervention and Post-Intervention periods. Prior day refusals were weighted and discarded daily before the morning feeding.

Cow Weight

All cows were weighted individually after the morning milking before new feed was administered using a PS-2000 scale (Salter Brecknell, Fairmont, MN) on the last day of Pre-Intervention period, and then on Intervention days 7, 14, 21, and 28; and Post-Intervention days 1, 6 and 10.

Milk Yield

Milk weighs were collected at each milking from ICAR approved Waikato MKV milk meters (Waikato, Hamilton, New Zealand) installed on each milking unit long milk hose.

Milk Sampling

Two composite milk samples per cow were collected at each milking on the last day of Pre-Intervention period, during the Intervention and Post-Intervention period. The Waikato Milk Meter retains a small percentage of the yield in a calibrated flask from which two milk samples were collected into 2 oz vials. One sample was analyzed using near-infrared spectroscopy (NIR) for crude protein, fat, and milk urea nitrogen (MUN) at the _____ (b) (4) The second sample was stored frozen at -20°C at _____ (b) (4) laboratory and shipped to Ascus Biosciences Laboratory at the end of the experiment.

Rumen Digesta Sampling

Rumen samples were collected once a day prior to inoculation after the morning milking on Intervention days 1, 2, 3, 5, 8, 11, 14, 17, 20, 23, 26, 29, and 32; and Post Intervention days 1, 4, 7 and 10. Two composite rumen samples were collected into 15 mL conicals from the dorsal, central, anterior and caudal parts of the rumen, consisting of both fluid and particulate. Rumen samples required the fixing of cells with 10% stock solution of 5% phenol and 95% ethanol. Conicals were sealed with parafilm and shipped frozen to Ascus Biosciences facility for microbial analysis.

Rumen pH

Rumen pH was measured on the last day of the Pre-Intervention period, and daily during the Intervention before inoculation and Post-Intervention periods. The rumen digesta was hand stirred and then scooped with a 13 mL vial. The pH was recorded immediately after ruminal fluid collection using a pH meter (Hanna Instruments, Woonsocket, RI).

TMR Sampling

One sample of TMR was collected on Pre-Intervention day 9, Intervention days 6, 13, 20, and 27; and Post-Intervention days 1, 5, and 9. TMR ingredients are reported in Table 2 and nutrient composition on Table 3. TMR samples were always collected one day before fecal sampling. TMR samples were collected using the quartering method at the different sampling times, stored frozen in vacuum-sealed bags and shipped to _____ (b) (4) at the end of the study to be analyzed using the NIR1 Plus Package. The NIR 1 Analysis includes tests for Dry Matter, Moisture, Crude Protein, ADF Protein, NDF Protein, Soluble Protein, ADF, NDF, NDFom, Lignin, Starch, Sugar, Fat, Ash, Calcium (Ca), Phosphorus (P), Magnesium (Mg), and Potassium (K). The NIR 1 Plus package in addition to what is evaluated in the NIR1 Package provides 30 hr NDF Digestibility with Kd Rate, NDF Digestibility at 120 and 240 hrs, uNDF120, and uNDF240.

Fecal Sampling

Feces were collected from the rectum using a palpation sleeve immediately after weighing the cows. Fecal samples were collected on the last day of the Pre-Intervention period, and then on Intervention days 7, 14, 21 and 28; and Post-Intervention days 2, 6 and 10. Approximately 55 g of feces was placed into 2 oz. vials, stored frozen and shipped at the end of the trial to _____ (b) (4) (b) (4) to be analyzed using the NIR1 Plus Package.

Outcomes Evaluated

Dry Matter Intake (DMI)

It is the feed consumed (Kg) in an as fed basis times the dry matter percentage of the feed obtained from the laboratory analysis The feed consumed was calculated by subtracting the amount of feed refused (not eaten) from the feed weight administered to cows on a daily basis.

Milk Yield

Daily milk yield was calculated as the sum of both morning and afternoon milk weights (Kg).

3.5% Fat Corrected Milk (FCM)

Milk yield value corrected for 3.5% fat using formula from NRC (2001): [(0.4324 × kg of milk) + (16.216 × kg of fat)].

Energy Corrected Milk (ECM)

Milk yield value corrected for 3.5% fat and 3.2% true protein using formula from NRC (2001): [(0.3246 × kg of milk) + (12.86 × kg of fat) + (7.04 × kg of true protein)].

Milk Components Percentage

Daily milk crude protein (%), fat (%), lactose (%), and MUN concentration (mg/dL) were calculated as the average of both morning and afternoon milk samples analysis results.

Milk Components Yield

Obtained multiplying daily milk crude protein (%), fat (%), lactose (%) and MUN (mg/dL) by the daily milk yield (Kg).

Feed Efficiency

Defined as Kg of 3.5% FCM produced per Kg of DM consumed.

Daily Body Weight Gain

Calculated as the difference in body weight between two measures divided by the number of days in between.

Rumen pH

pH reading from the days which was measured.

Fecal Matter

It was evaluated dry matter (DM), starch, NDF, protein, and lignin.

Apparent Nutrient Digestibility

Includes a NIR Plus evaluation of feed and associated fecal matter to generate an evaluation of apparent nutrient digestibility. In order to calculate nutrient digestibility 240-hr in vitro digestion is was performed and undigested NDF at 240 hr (uNDFom240) is used as a marker. It assumes the amount of uNDFom240 is constant in both the feces and the feed so the relative differences between the feed and feces will give the estimate of digestibility. It allows to determine the amount of CP, NDF and starch in the manure without having to measure the quantity of manure cows are producing.

Study Incidences

During the Pre-Intervention period, Cow 10 which was assigned to Control had a displaced abomasum, which negatively led to a loss of appetite, drop in milk yield and mild diarrhea. The sick animal was removed from the study and data from this cow was not used in the analysis. This cow was replaced by another cow on January 30th, 2016 (Intervention day 3) and data from this cow was used in the analysis.

In addition, cows with study IDs 8, 14, 16 had health problems (fever, displaced abomasum, etc) with episodes of anorexia and low milk production. Finally, cows 3 and 7 although healthy produced less milk than expected due to a large daily variation in milk production.

Statistical Analysis and Results Layout

Milk production, milk composition, body weight gain and rumen pH were measured daily on 16 cows for 32 days during treatment application and another 10 days after inoculation. Fecal nutrients concentration and nutrients apparent digestibility were measured by pooling two cows within the same treatment group such that 8 experimental units were available for analysis. Therefore, the present report is structured in three sections: 1) The first section (SECTION I) presents the results of the statistical analysis of dry matter intake (DMI), milk production, milk composition, body weight gain and rumen pH during the Intervention period; 2) The second section (SECTION II) includes graphical representation of dry matter intake, milk production, milk composition, body weight gain and rumen pH during the Intervention and Post-Intervention periods; and, 3) The third section (SECTION III) presents the results of the statistical analysis of digestibility.

SECTION I: Dry Matter Intake, Milk Production and Composition, Body Weight Gain and Rumen pH During the Intervention Period

Statistical Analysis

Data was analyzed using the SAS/STAT software, Version 9.3 of the SAS System for PC. Copyright © 2014 SAS Institute Inc., Cary, NC, USA. Daily values were originally analyzed implementing random coefficients models with linear and quadratic terms. However, due to the small sample size and the model complexity, for several of the outcomes the model convergence was not obtained. Therefore, daily values were averaged to produce weekly means. Week 5 averages included only 4 days while the remaining weeks included 7 daily values. Weekly DMI, milk yield, milk composition, body weight gain and rumen pH were analyzed as repeated measures using the MIXED procedure available within SAS/STAT software. The model included the fixed effect of treatment (Control vs. Inoculated), time (week 1, 2, 3, 4 and 5) and their interaction. Milk yield and DMI measured the three days prior to treatment application, were averaged and used as covariate for the corresponding outcome variable. Cow within treatment was the subject of the repeated statement. The covariance structure that provided the best fit according to the Bayes Information Criterion (BIC) was chosen. The covariance structure employed consisted of unstructured for DMI, milk protein and lactose percentages and fat yield, compound symmetry for milk urea nitrogen, and first order autoregressive for the remaining outcomes. Furthermore, where appropriate separate residual variances for each treatment were estimated as they provided a better fit according to BIC. When a significant treatment by time interaction was observed, treatment means within week were compared using the SLICE option. Significance was declared at p-value <0.05 and tendency was declared at 0.05≤ p-value <0.10.

A total of two analyses were conducted on the collected data: 1. The first analysis (n=16) included all collected observation on all cows; and, 2. The second analysis (n=11) excluded three cows (study IDs 8, 14 and 16) from Control that had health events and two cows from Intervention (study IDs 3 and 7) because of large daily milk production variability. All the analyses were executed using the previously described models, except that for analyses two the covariance structure for the repeated measures was reassessed. The covariance structure employed consisted of unstructured for feed efficiency, compound symmetry for fat percentage and milk urea nitrogen, and first order autoregressive for the remaining outcomes. Analysis 1 is reported in the Results section while analyses 2 is reported as Appendix B.

Results

Treatment least square means, fixed effects and covariance parameters estimates of the analysis including all cows (analysis 1) are reported in Table I-1 and Figures I-1 to I-13. A treatment by week interaction was observed for milk yield (P = 0.0025, Figure I-2), FCM (P = 0.0026, Figure I-3), ECM (P = 0.0019, Figure I-4), and protein yield (P = 0.0012, Figure I-8). A tendency for a treatment by week interaction was also observed for fat yield (P = 0.0880, Figure I-9), feed efficiency (FE, P = 0.0671, Figure I-11) and rumen pH (P = 0. 0741, Figure I-13). The interaction for yields was mainly the result of milk yield diverging between the two treatments within the first 2-3 weeks of the study, but not toward the end of the Intervention period.

A tendency for a higher milk fat percentage for Inoculated vs. the Control was observed (P = 0.0991). Although the treatment by week interaction was not significant (P = 0.2677, Figure I-6), it can be observed that milk fat percentages were numerically similar within the first two weeks and numerically higher for Intervention during weeks three to five. No other main effect was either significant or tended to be significant without also having a significant treatment by week effect.

Comment: The statistical analysis performed included all the weekly means when the treatment was applied; as such treatment by time interactions should be the main focus. Treatment main effects and least square means included the weekly values at the beginning of the Intervention period when cows still not responded to treatment due to adaptation. Furthermore, as the number of cows was not very large the main focus should be effect size and not the lack or presence of statistical significance.

Table I-1: Dry matter intake, milk production and composition, BW gain and rumen pH least square means (± SEM) of cows assigned to Control and Inoculated.

Outcome	Treatment		Fixed Effects[1]			
	Control	Inoculated	Cov	Tx	Week	Tx*Week
DMI, kg	26.2 ± 2.8	30.2 ± 1.2				
Milk yield, kg	25.7 ± 1.9	30.6 ± 1.9				
FCM, kg	27.7 ± 2.5	32.5 ± 2.5				
ECM, kg	27.2 ± 2.4	32.1 ± 2.4				
Milk components, %						
Crude Protein	3.08 ± 0.06	3.27 ± 0.11				
Fat	3.87 ± 0.08	4.06 ± 0.08				
Lactose	4.64 ± 0.10	4.73 ± 0.03				
Milk components yield, kg						
Crude Protein	0.80 ± 0.07	0.97 ± 0.07				
Fat	1.01 ± 0.10	1.20 ± 0.10				
MUN, mg/dL	6.17 ± 0.60	7.41 ± 0.45				
FCM/DMI	1.22 ± 0.07	1.10 ± 0.07				
BW gain, kg/day	0.78 ± 0.44	1.46 ± 0.43				
Rumen pH	6.24 ± 0.09	6.05 ± 0.09				

(b) (4)

[1]Cov= covariate effect, Tx = treatment effect, Day = day effect; Tx*Day = treatment by day interaction.

Figure I-1: Dry matter intake (kg) daily means (no fill) and covariate adjusted weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 2201) and treatment by time interaction (P = 0.1910). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).
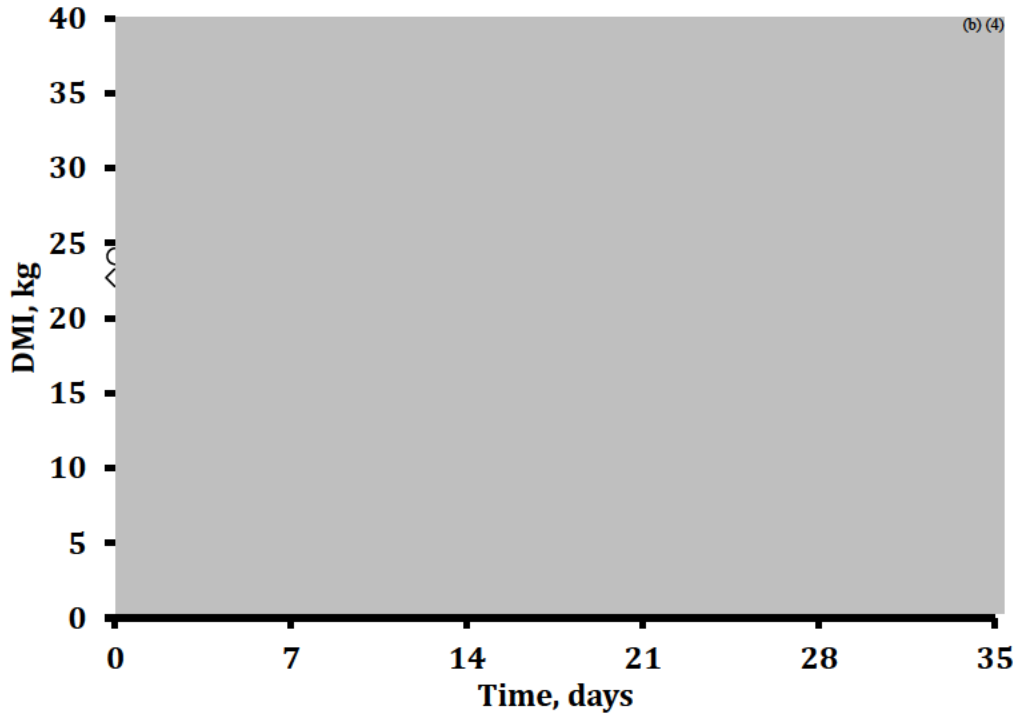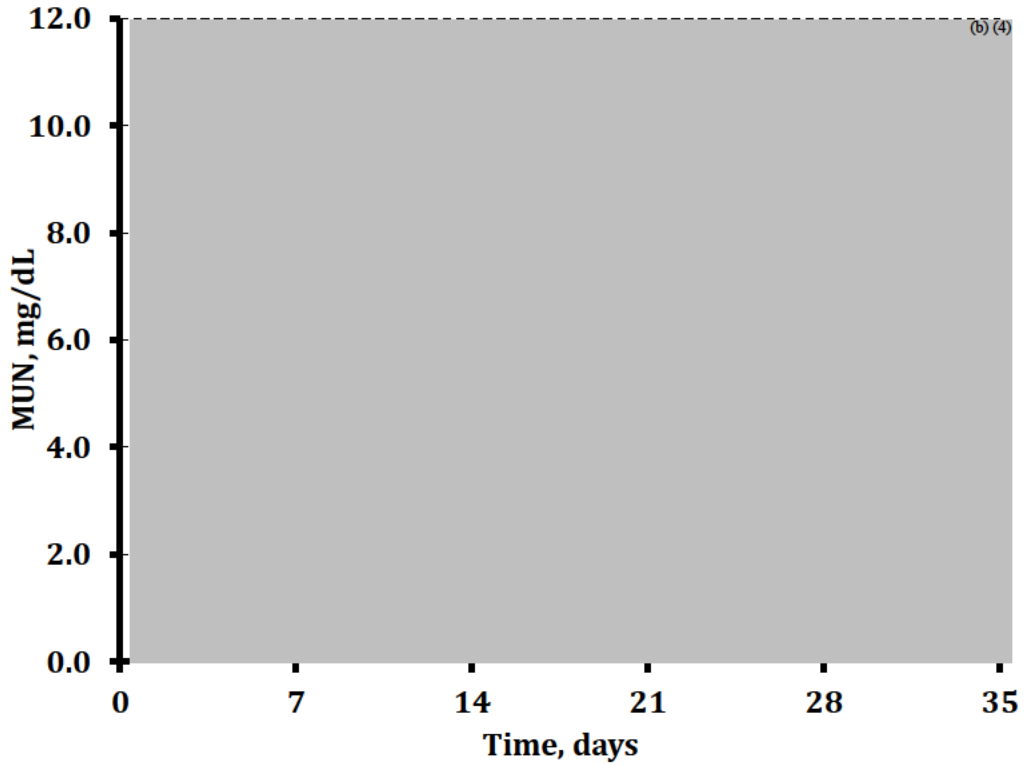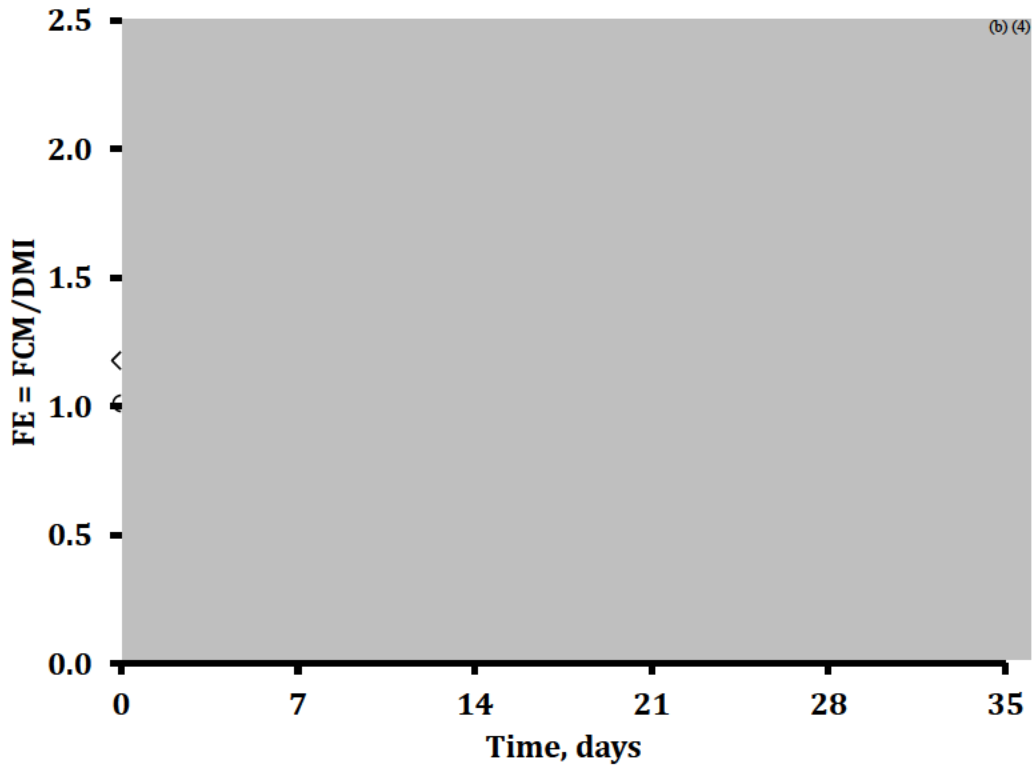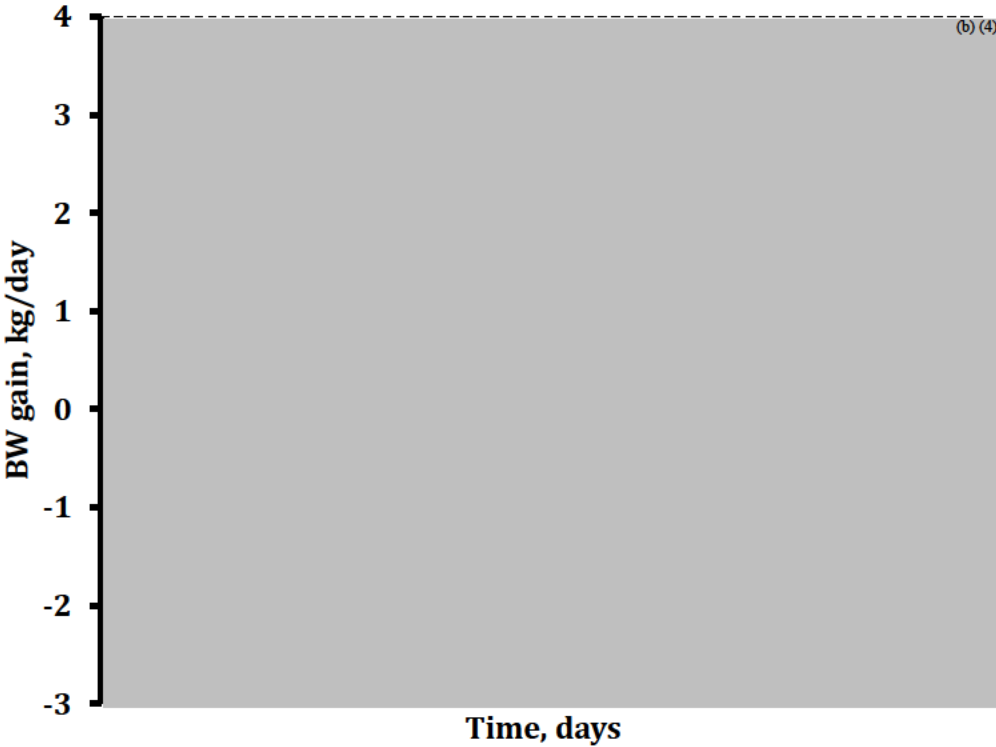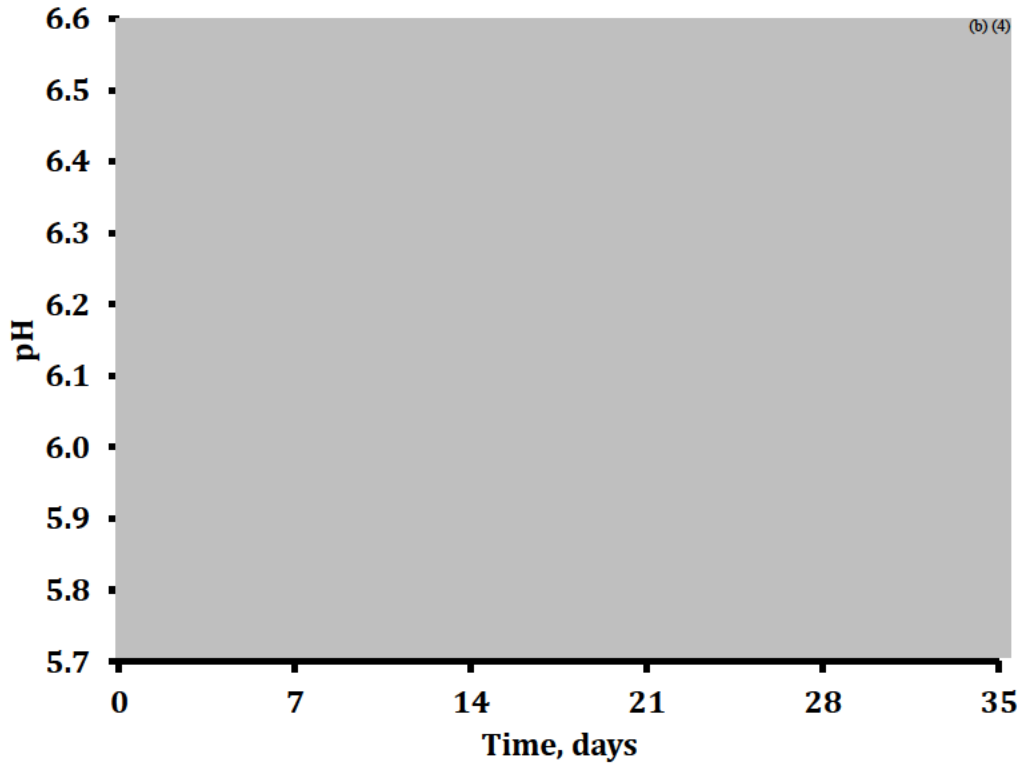
Figure I-2: Milk yield (kg) daily means (no fill) and covariate adjusted weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.0791$) and treatment by time interaction ($P = 0.0025$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).
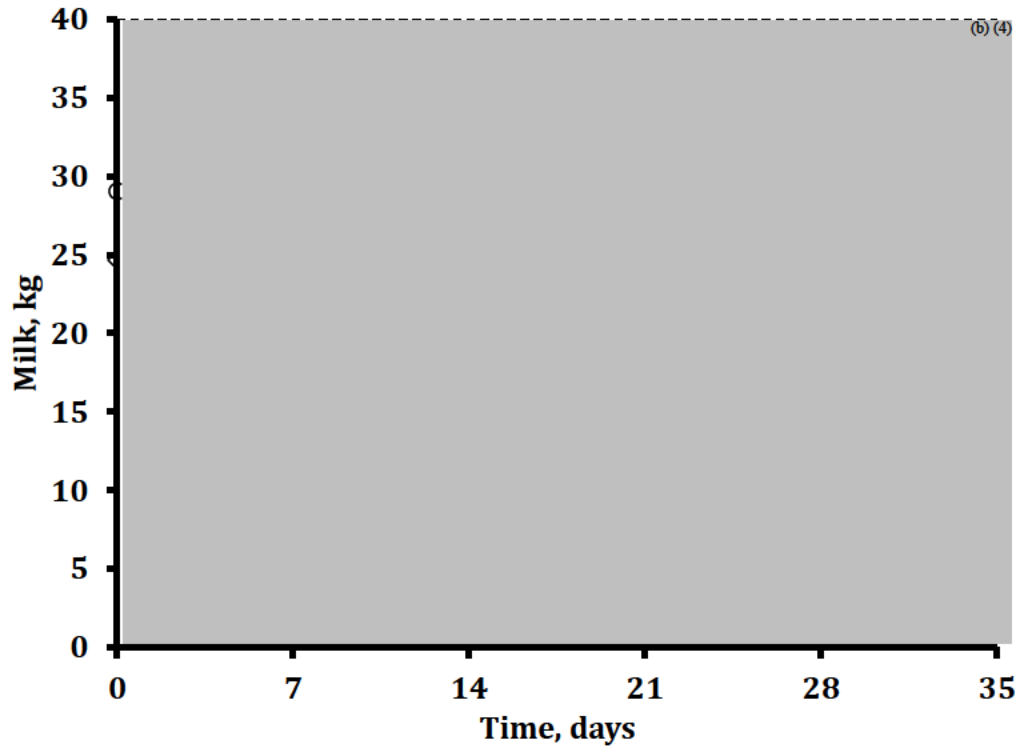
Figure I-3: Fat corrected milk yield (FCM, kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1883) and treatment by time interaction (P = 0.0026). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).
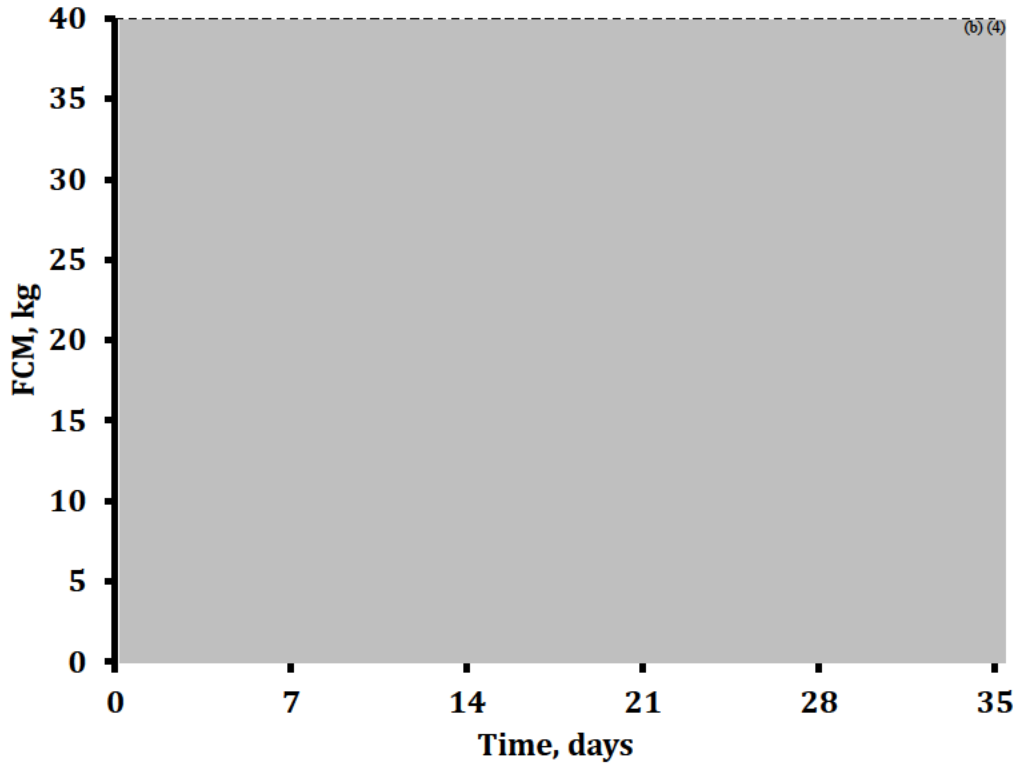
Figure I-4: Energy corrected milk yield (ECM, kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1669) and treatment by time interaction (P = 0.0019). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).
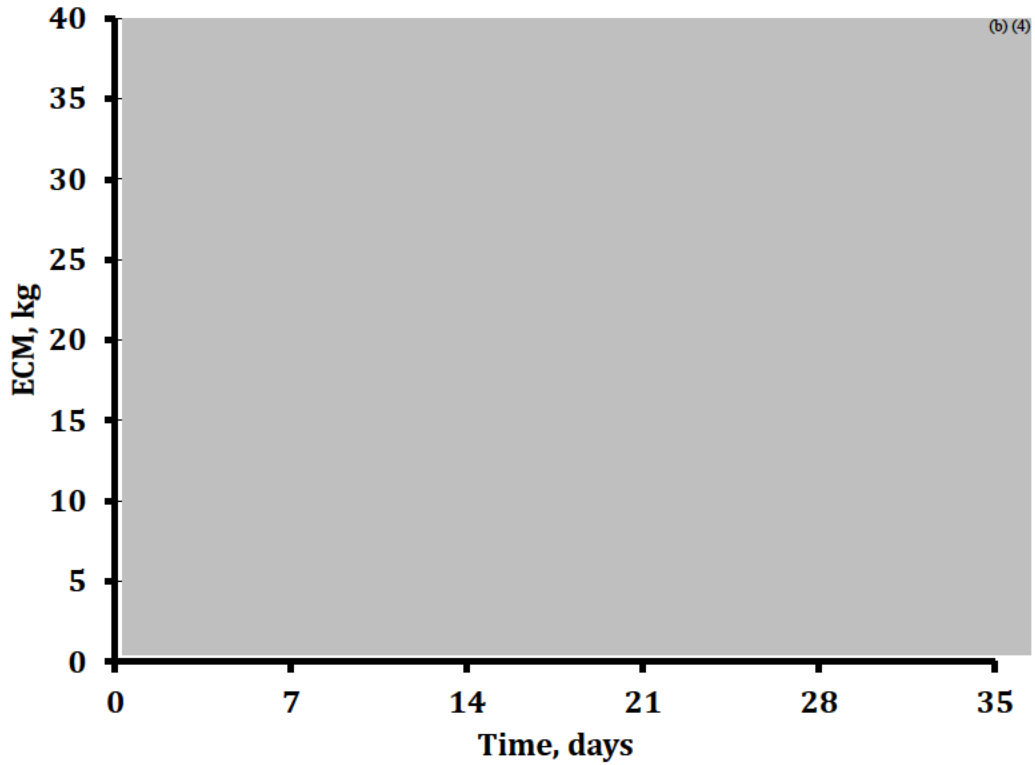
Figure I-5: Milk crude protein (CP, %) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0.1553) and treatment by time interaction (*P* = 0.3125). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).
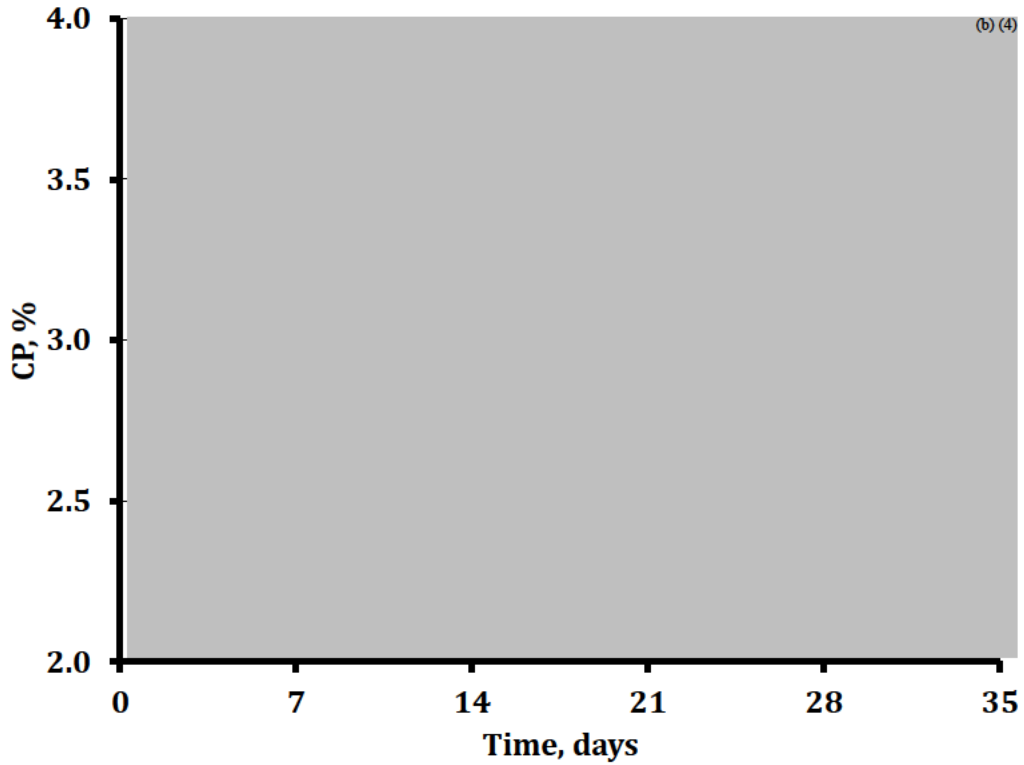
Figure I-6: Milk fat (%) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.0991$) and treatment by time interaction ($P = 0.2677$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).
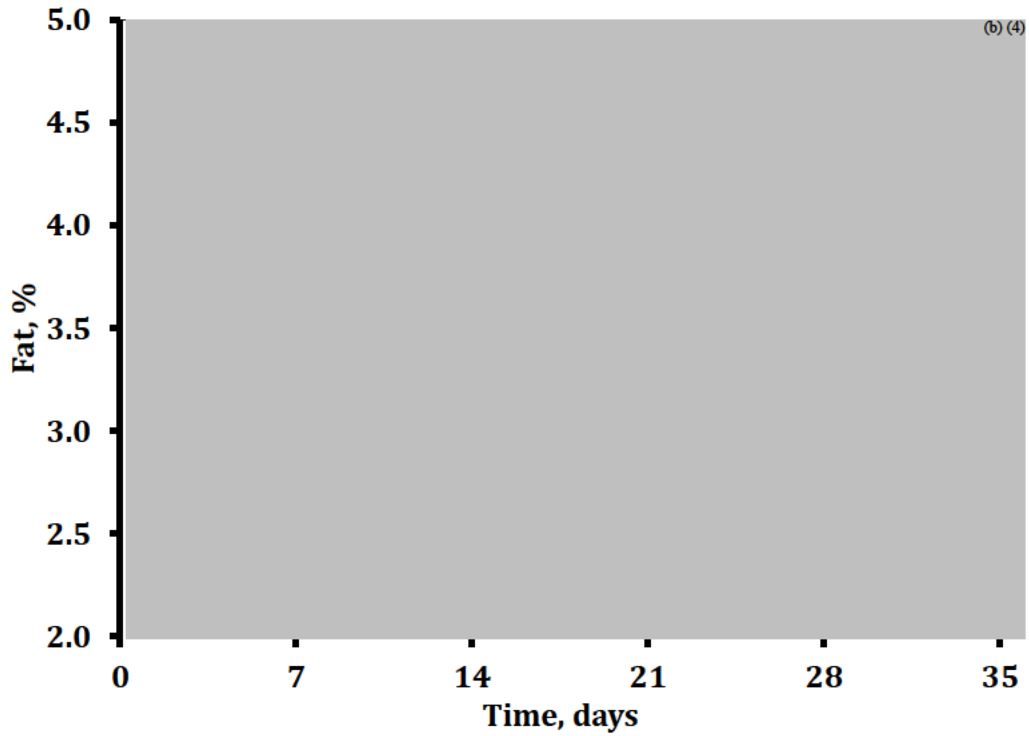
Figure I-7: Milk lactose (%) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.3787) and treatment by time interaction (P = 0.5016). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).
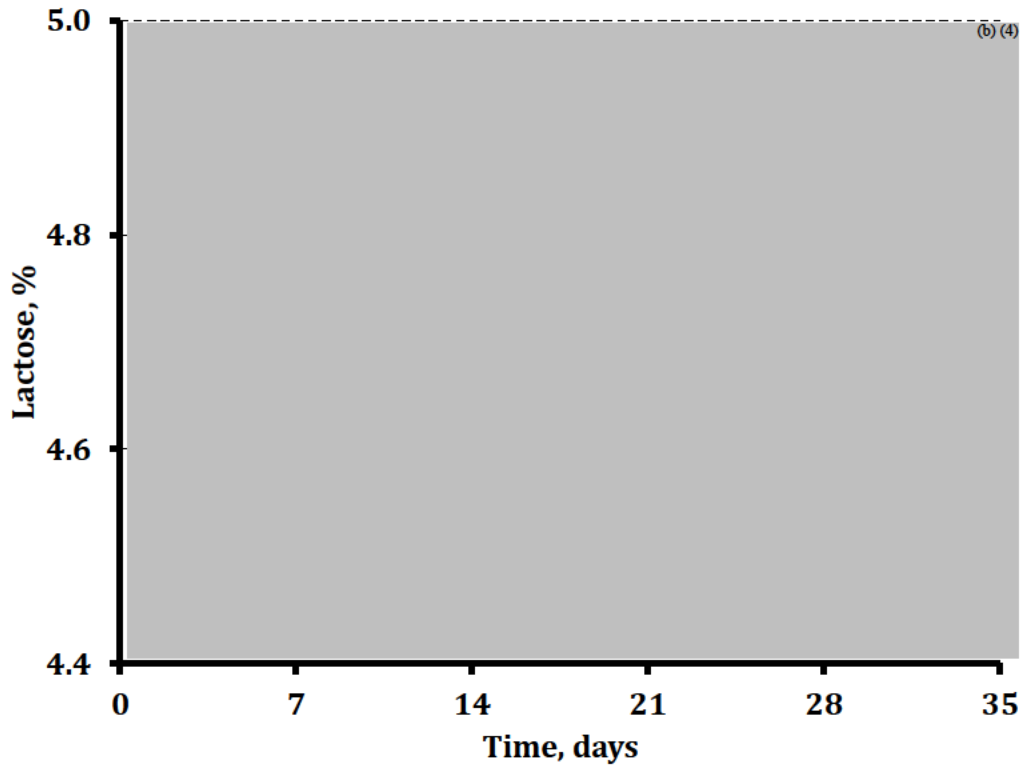
Figure I-8: Milk crude protein yield (CP, kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1183$) and treatment by time interaction ($P = 0.0012$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).
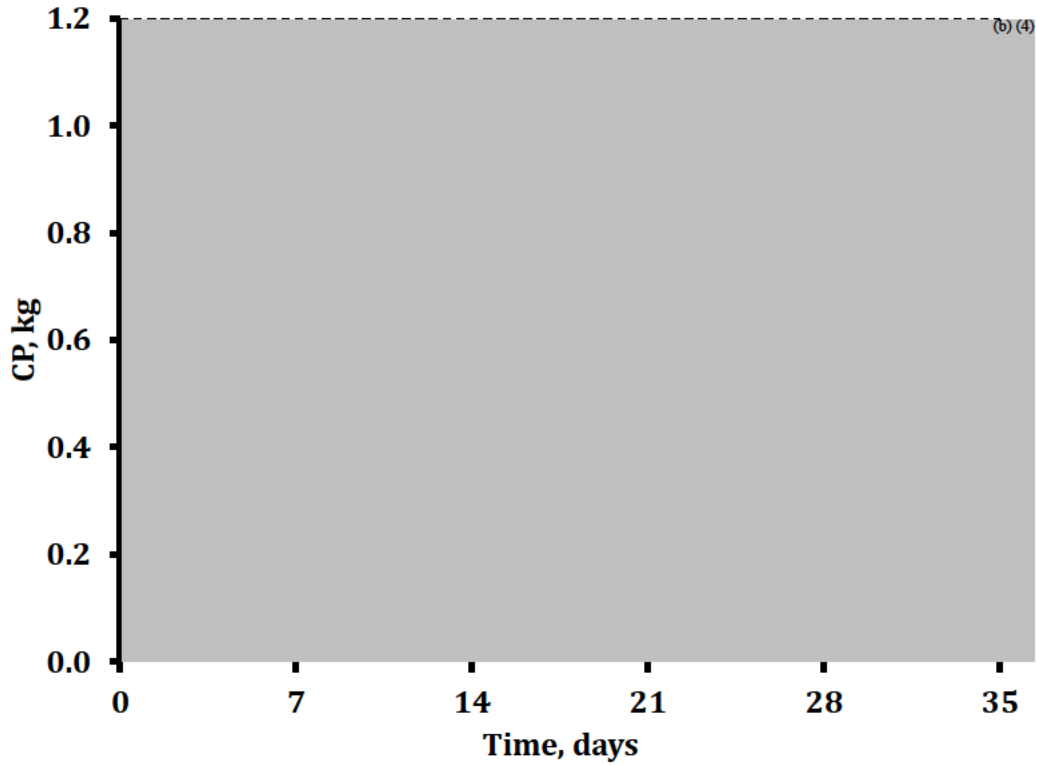
Figure I-9: Milk fat yield (kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1818) and treatment by time interaction (P = 0.0880). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).
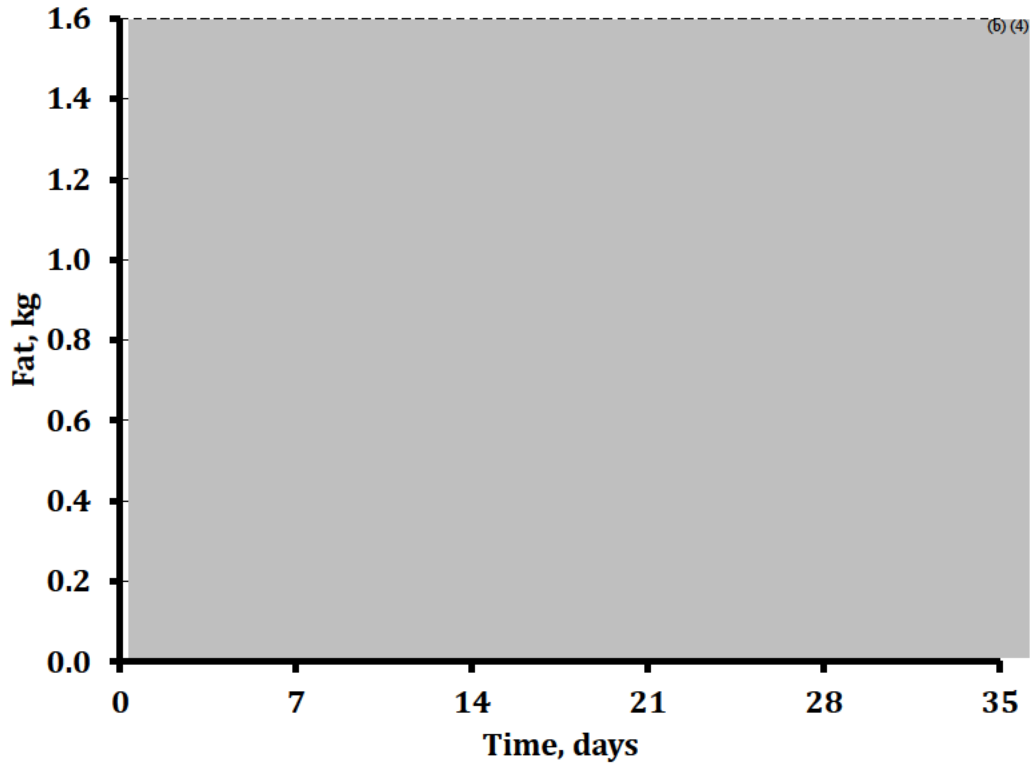
Figure I-10: Milk urea nitrogen (MUN, mg/dL) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1222$) and treatment by time interaction ($P = 0.3440$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).

Figure I-11: Feed efficiency (FCM/DMI) means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.2835) and treatment by time interaction (P = 0.0671). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

Figure I-12: BW gain (kg/day) weekly least square means± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.2838$) and treatment by time interaction ($P = 0.3335$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).

Figure I-13: Rumen pH daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1600$) and treatment by time interaction ($P = 0.0741$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).

SECTION II: Dry Matter Intake, Milk Production and Composition, Body Weight Gain and Rumen pH During the Intervention and Post-Intervention Periods

As previously stated, the following section reports SECTION 1 figures with added on a graphical representation of the production portion of the study once the supplementation ended.

Figure II-1: Dry matter intake (kg) daily means (no fill), covariate adjusted weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 2201) and treatment by time interaction (P = 0.1910). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01). The vertical line represents the end of the feeding trial.
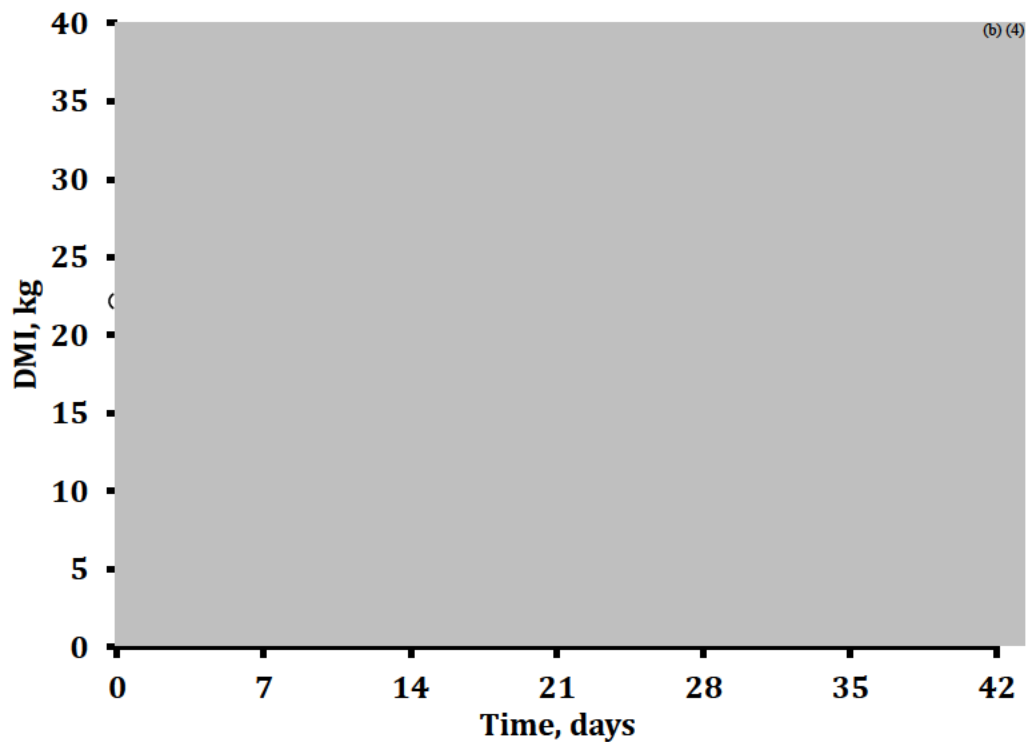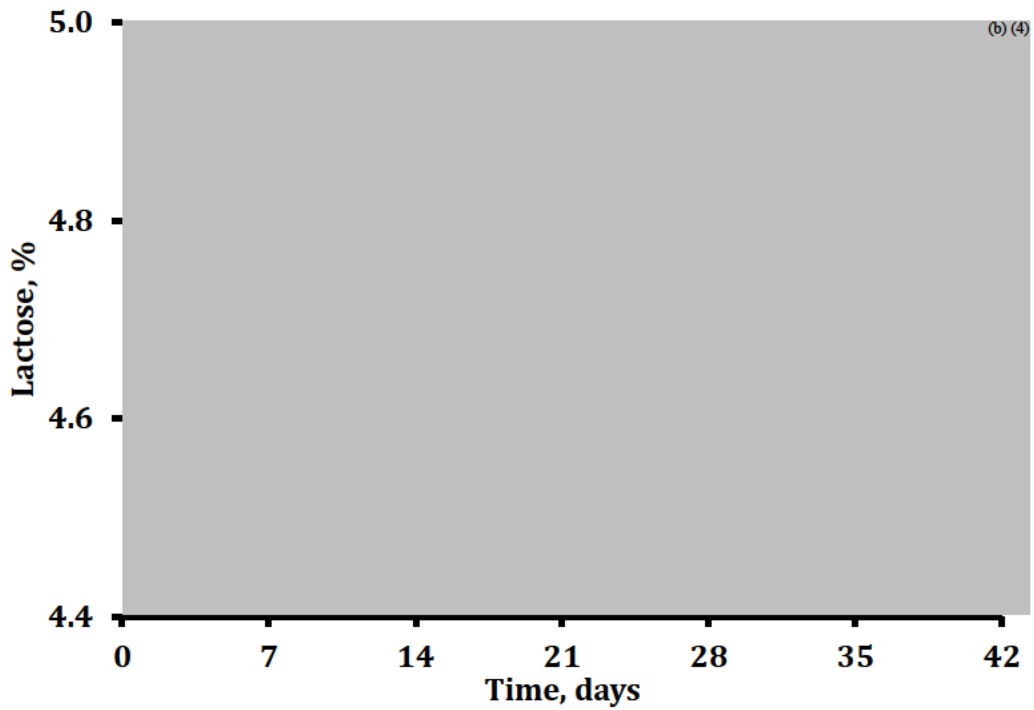
Figure II-2: Milk yield (kg) daily means (no fill), covariate adjusted weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.0791$) and treatment by time interaction ($P = 0.0025$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.
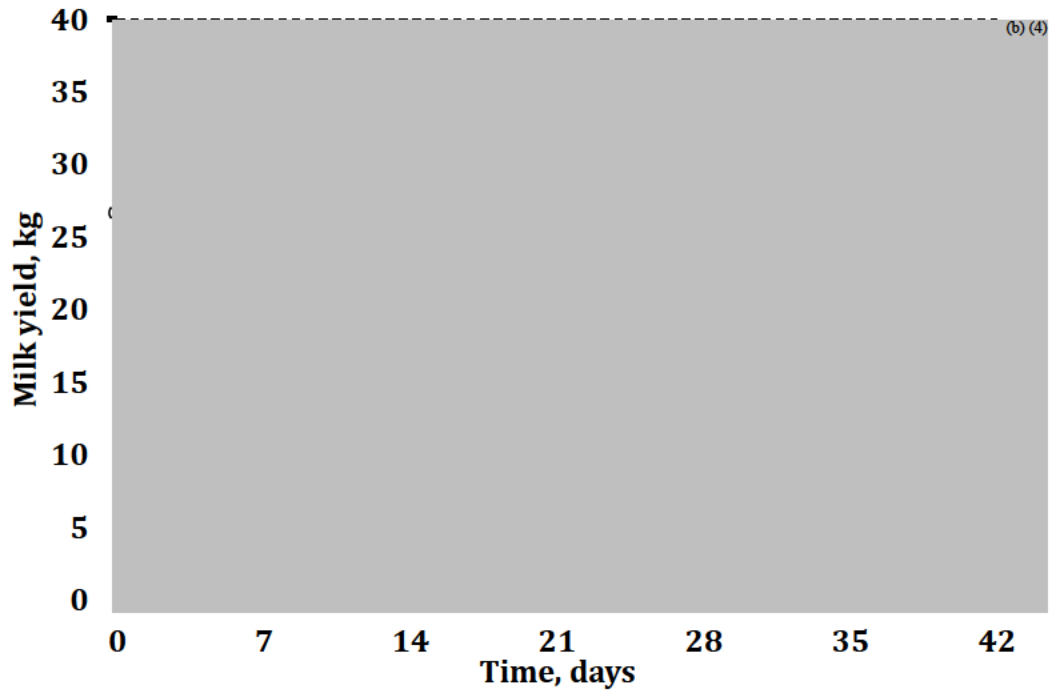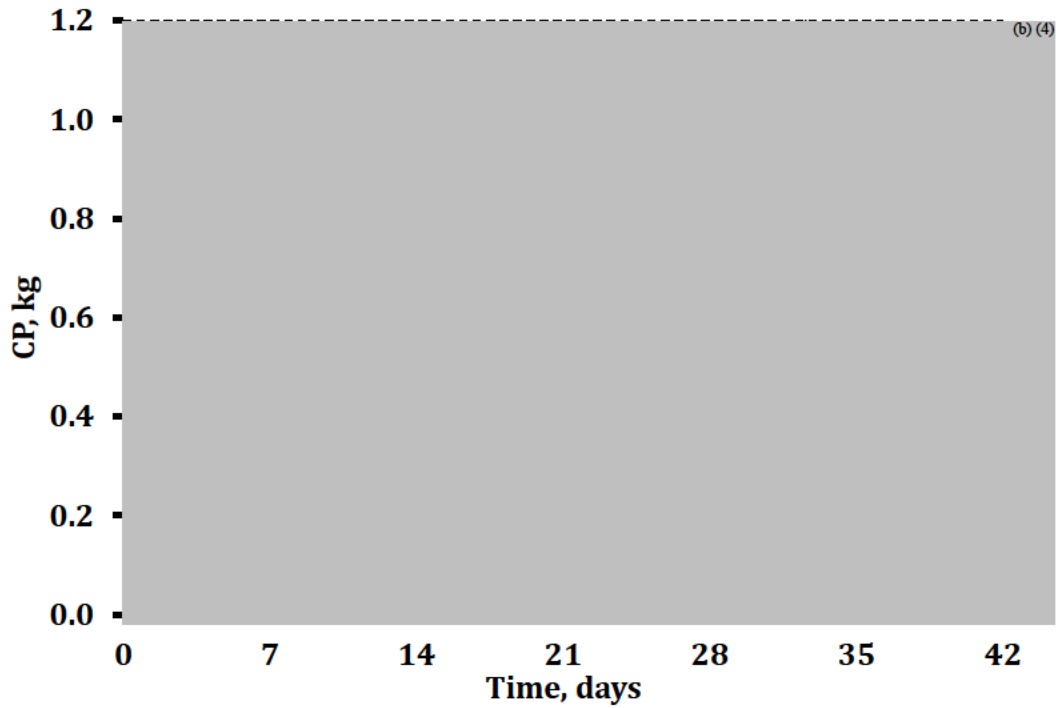
Figure II-3: Fat corrected milk yield (FCM, kg) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1883) and treatment by time interaction (P = 0.0026). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01). The vertical line represents the end of the feeding trial.
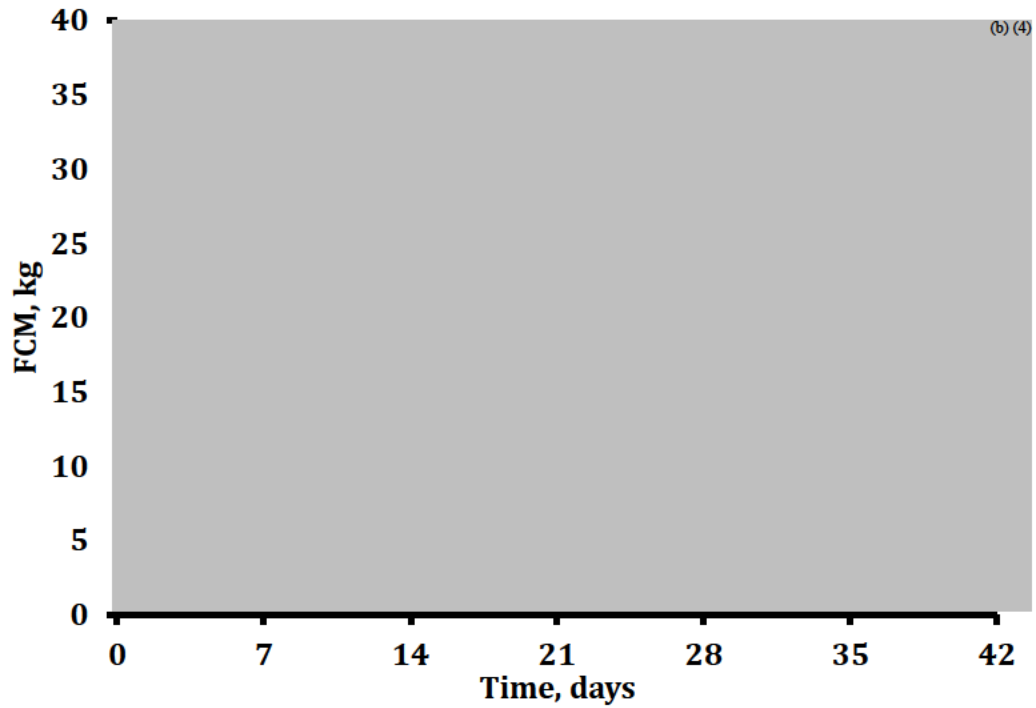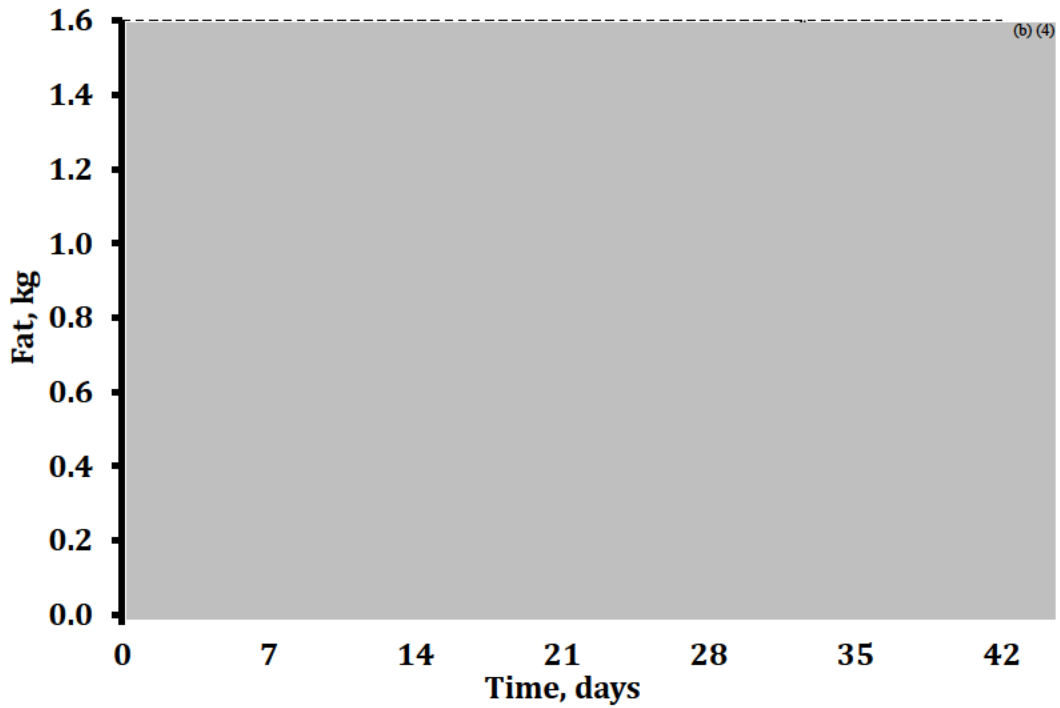
Figure II-4: Energy corrected milk yield (ECM, kg) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1669$) and treatment by time interaction ($P = 0.0019$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.
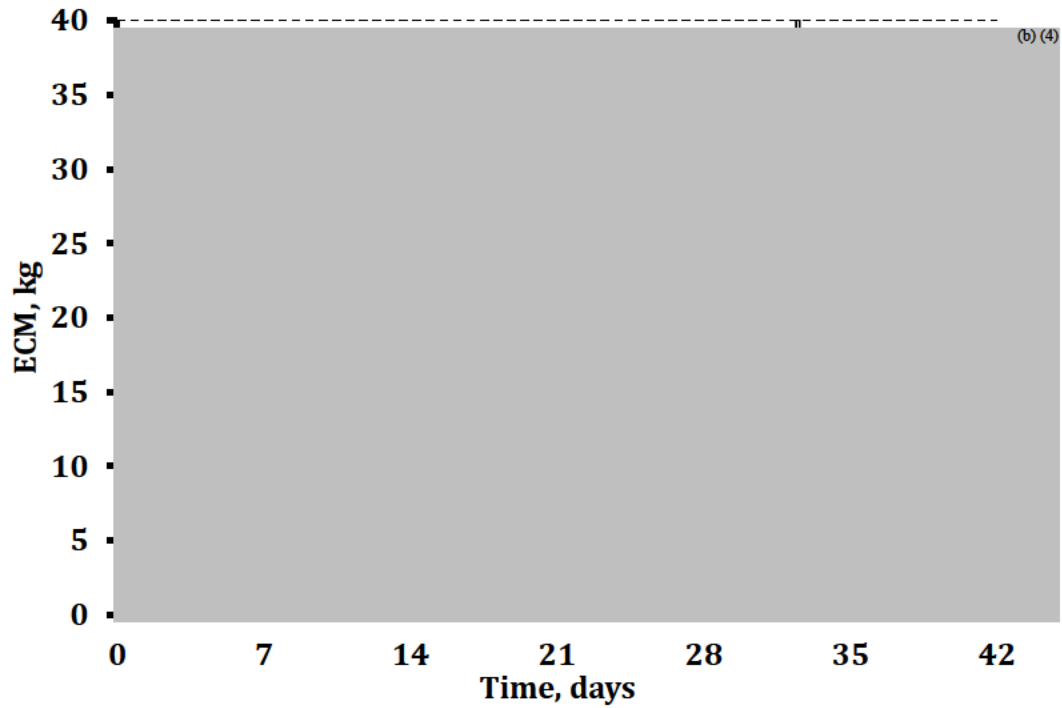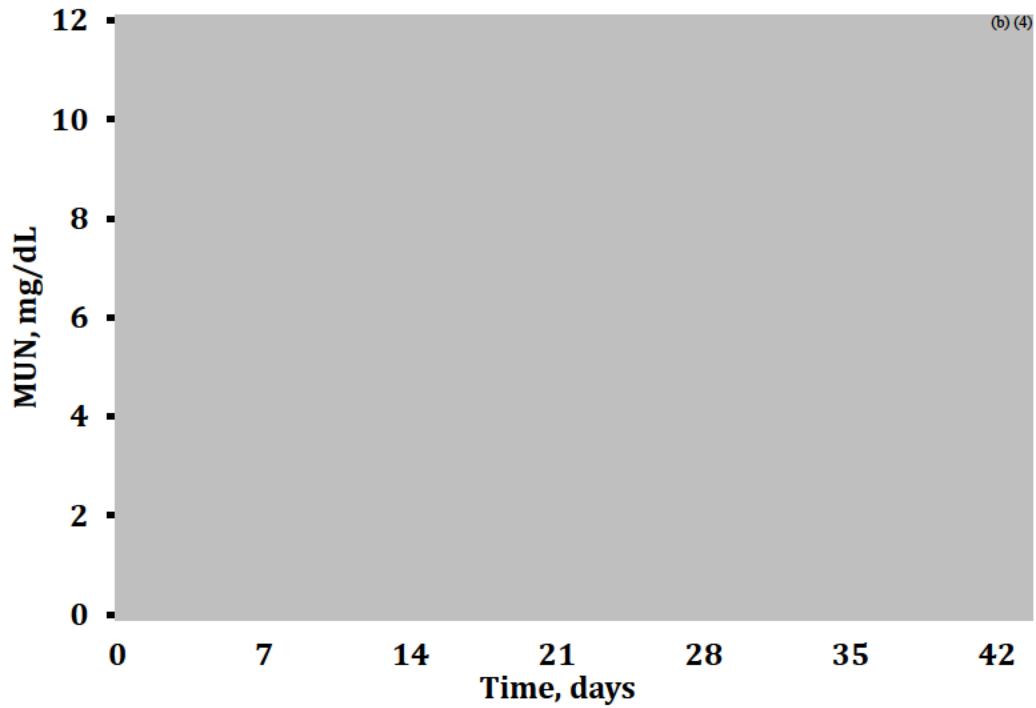
Figure II-5: Milk crude protein (CP, %) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1553$) and treatment by time interaction ($P = 0.3125$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.
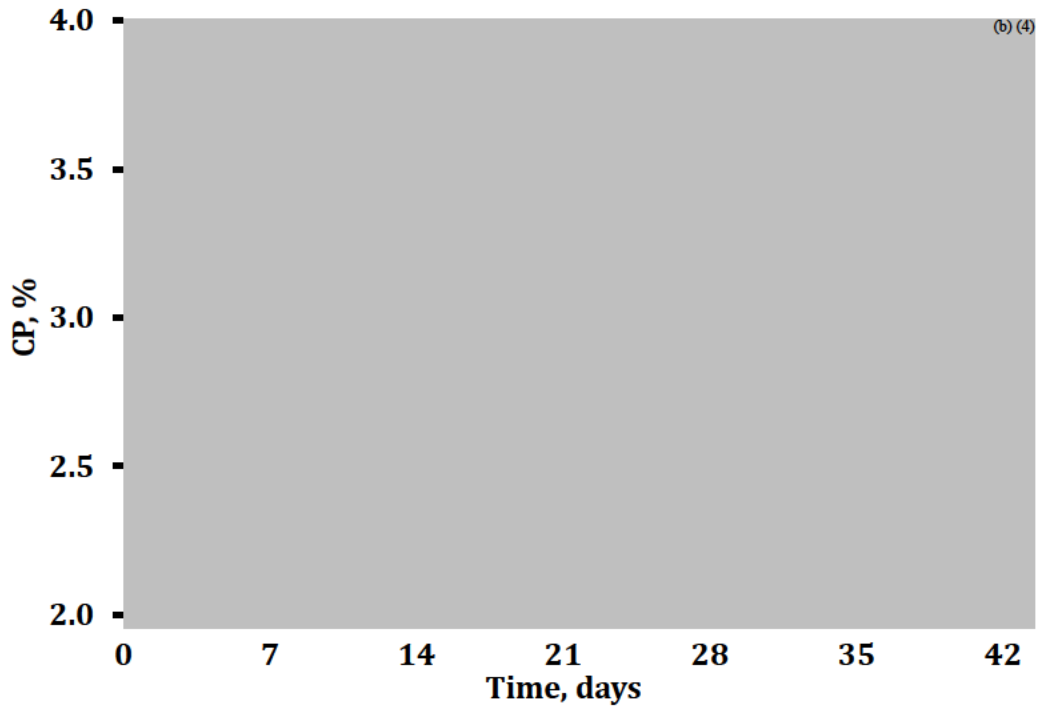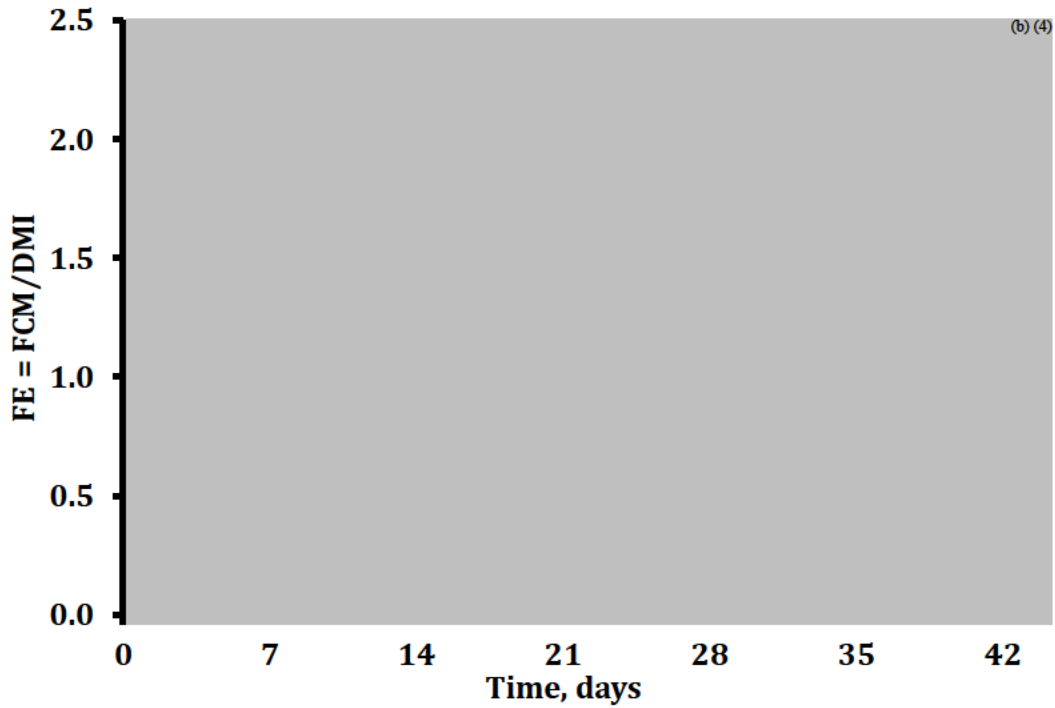
Figure II-6: Milk fat (%) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.0991$) and treatment by time interaction ($P = 0.2677$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.
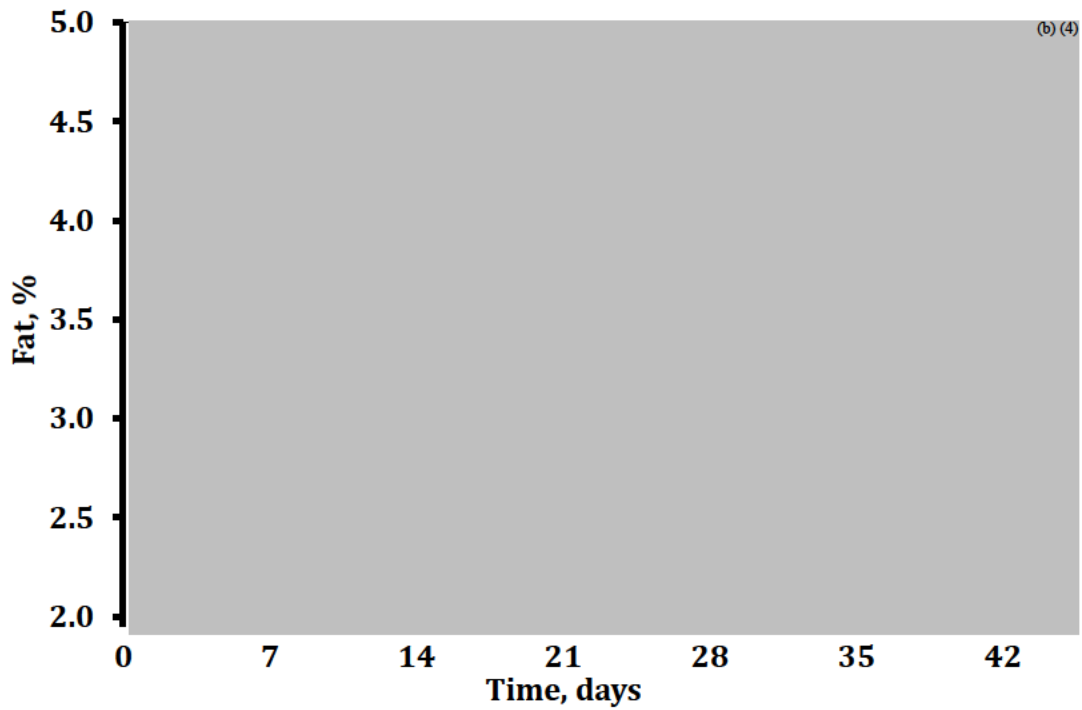
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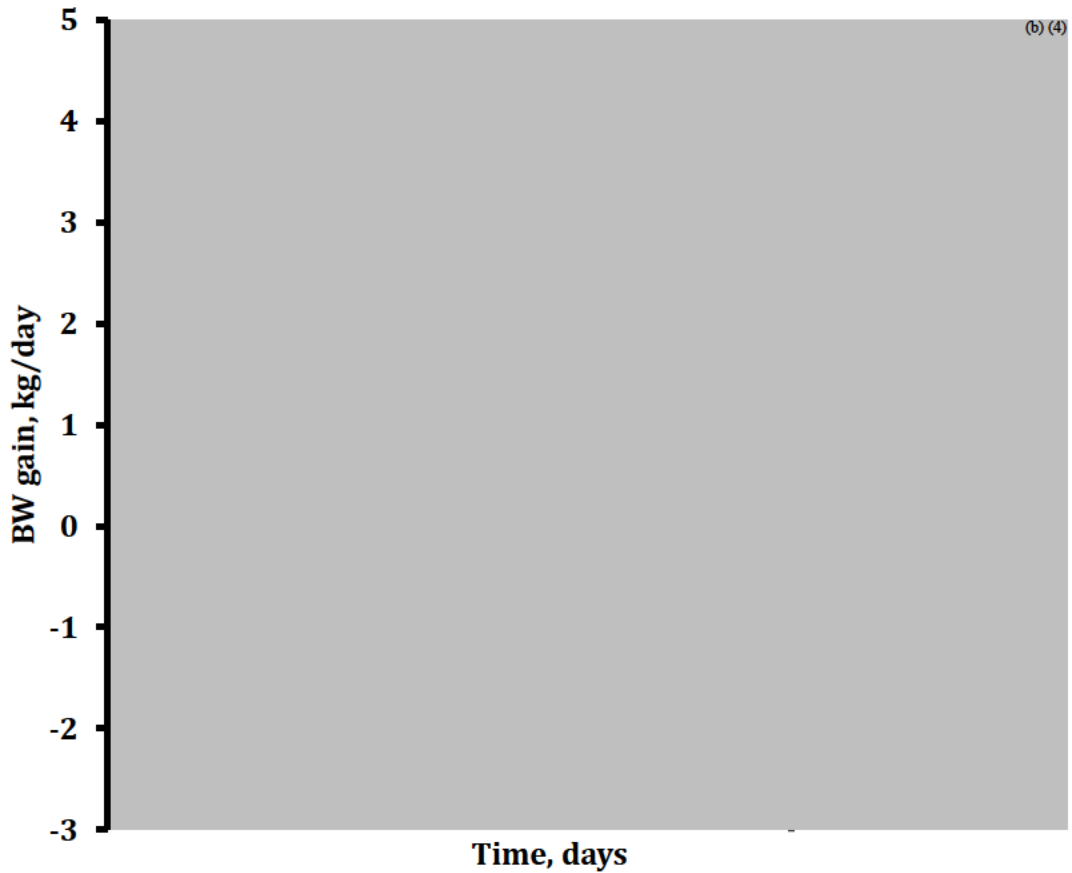
Figure II-8: Milk crude protein yield (CP, kg) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1183$) and treatment by time interaction ($P = 0.0012$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.

Figure II-9: Milk fat yield (kg) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1818$) and treatment by time interaction ($P = 0.0880$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.

Figure II-10: Milk urea nitrogen (MUN, mg/dL) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1222) and treatment by time interaction (P = 0.3440). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01). The vertical line represents the end of the feeding trial.

Figure II-11: Feed efficiency (FCM/DMI) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.2835$) and treatment by time interaction ($P = 0.0671$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.

Figure II-12: BW gain (kg/day) weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) weekly least square means± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.2838$) and treatment by time interaction ($P = 0.3335$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.

Figure II-13: Rumen pH daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1600) and treatment by time interaction (P = 0.0741). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01). The vertical line represents the end of the feeding trial.
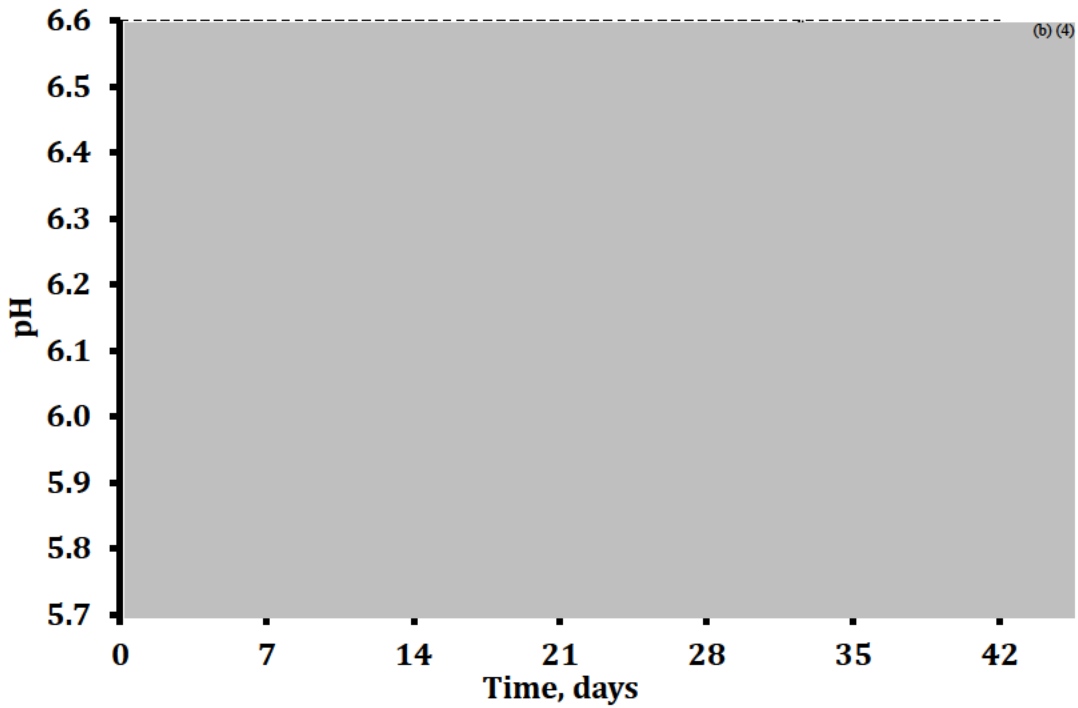
SECTION III: Nutrient Composition of Feces and Digestibility

Statistical Analysis

Data was analyzed using the SAS/STAT software, Version 9.3 of the SAS System for PC. Copyright © 2014 SAS Institute Inc., Cary, NC, USA. Weekly fecal nutrients concentration and apparent nutrients digestibility were analyzed as repeated measures using the MIXED procedure available within SAS/STAT software. The model included the fixed effect of treatment (Control vs. Inoculated), time (week 1, 2, 3, 4 and 5) and their interaction. Measurements collected prior to treatment application were used as a covariate for the corresponding outcome variable. Unit ID within treatment was the subject of the repeated statement. The covariance structure that provided the best fit according to the Bayes Information Criterion (BIC) was chosen. The covariance structure employed consisted of compound symmetry for fecal percentage of DM, starch, NDF and protein and unstructured for the remaining outcomes. When a significant treatment by time interaction was observed, treatment means within week were compared using the SLICE option. Significance was declared at p-value <0.05 and tendency was declared at 0.05≤ p-value <0.10.

Results

Treatment least square means, fixed effects and covariance parameters estimates of the analysis including all units (analysis 1) are reported in Table III-1 and Figures III-1 to III-8. No significant treatment by week or main effect of treatment was observed on any of the outcomes measured. Fecal starch percentage tended to be higher for Inoculated vs Control (P = 0.0714) and consequently also a tendency for a lower starch digestibility for Inoculated was observed (P = 0. 0745).

Table III-1: Fecal matter concentration and digestibility least square means of cows assigned either to control or Inoculated.

Outcome	Control	Inoculated	SEM	Cov	Tx	Week	Tx*Week
Fecal matter, %							(b) (4)
DM	15.8	15.9	0.4				
Starch	5.4	7.2	0.5				
NDF	52.0	51.6	0.5				
Protein	19.5	19.3	0.5				
Lignin	11.6	10.8	0.4				
Digestibility							
Starch	89.6	86.8	0.9				
NDF	22.2	18.7	2.0				
Protein	54.2	53.2	1.5				

[1]Cov= covariate effect, Tx = treatment effect, Day = day effect; Tx*Day = treatment by day interaction.

Figure III-1: Fecal DM (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 9170) and treatment by time interaction (P = 0.6705). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).
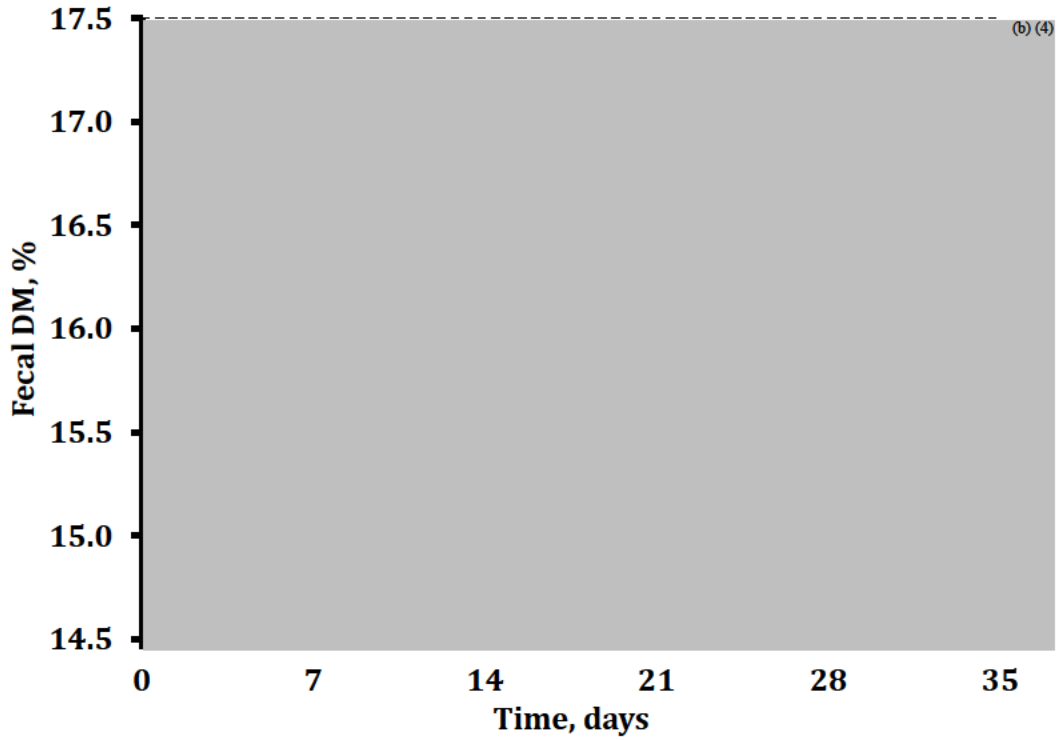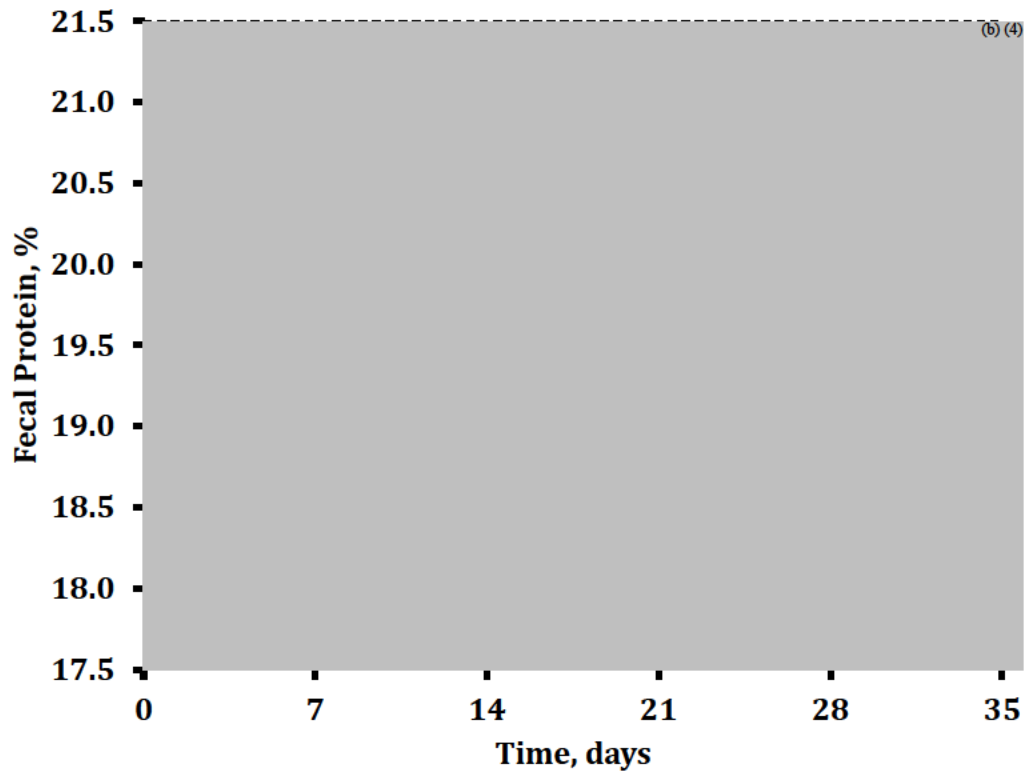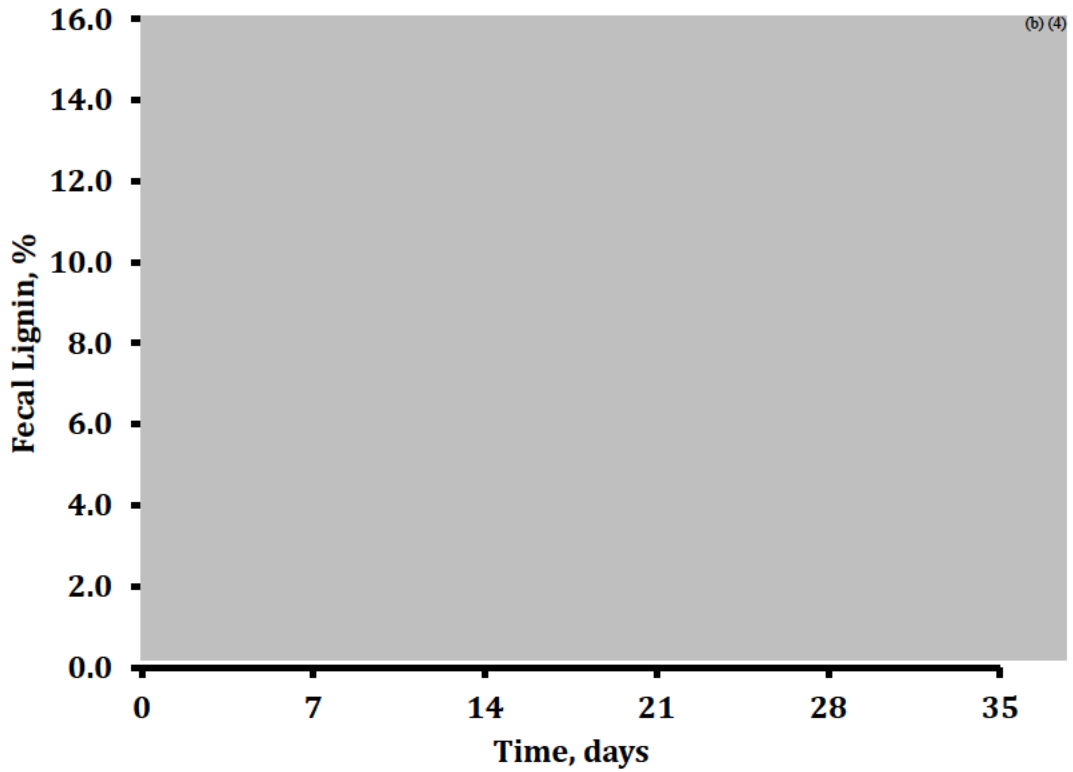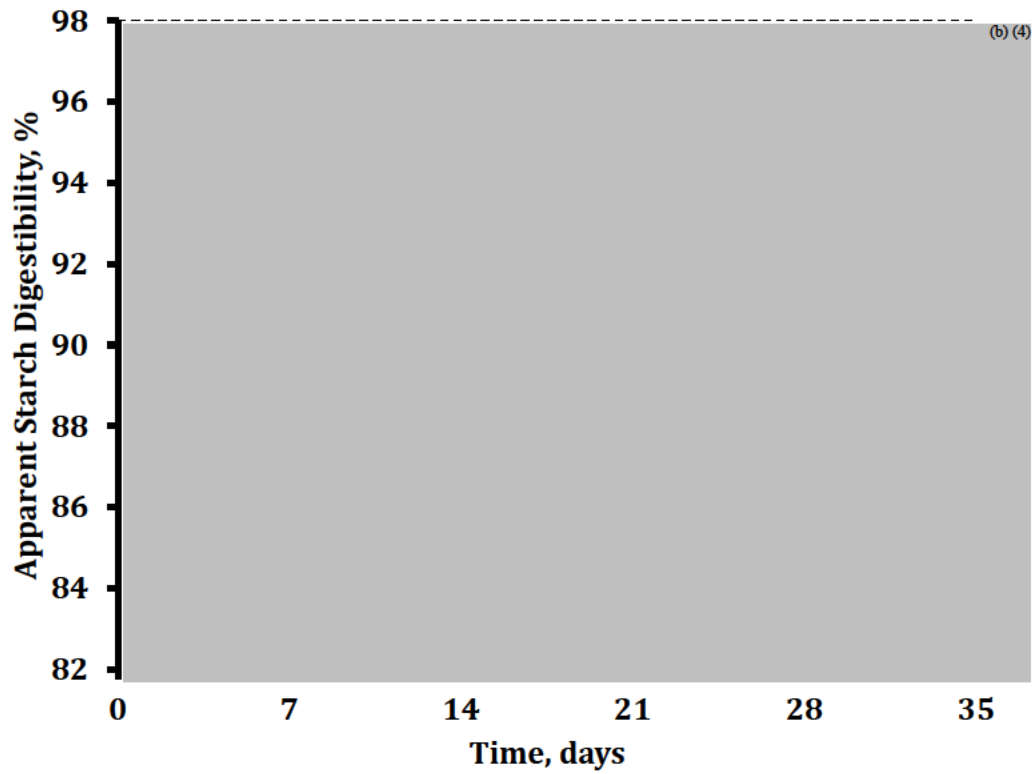
Figure III-2: Fecal Starch (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 0714) and treatment by time interaction (P = 0.2842). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).
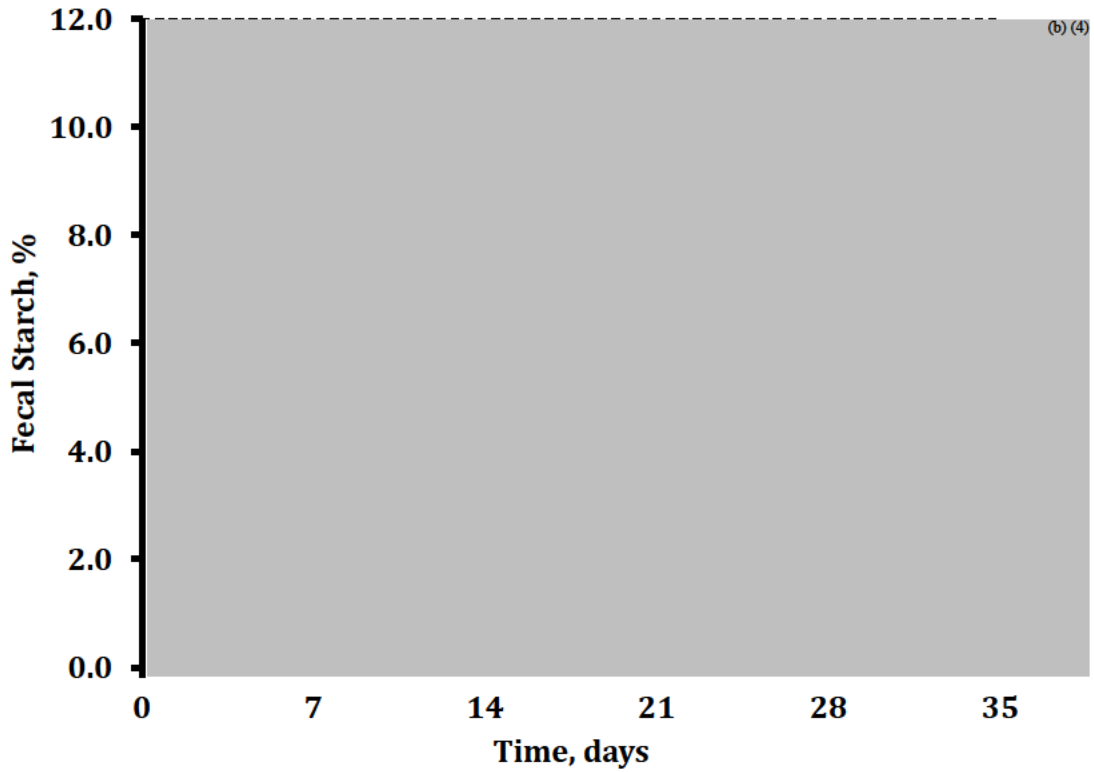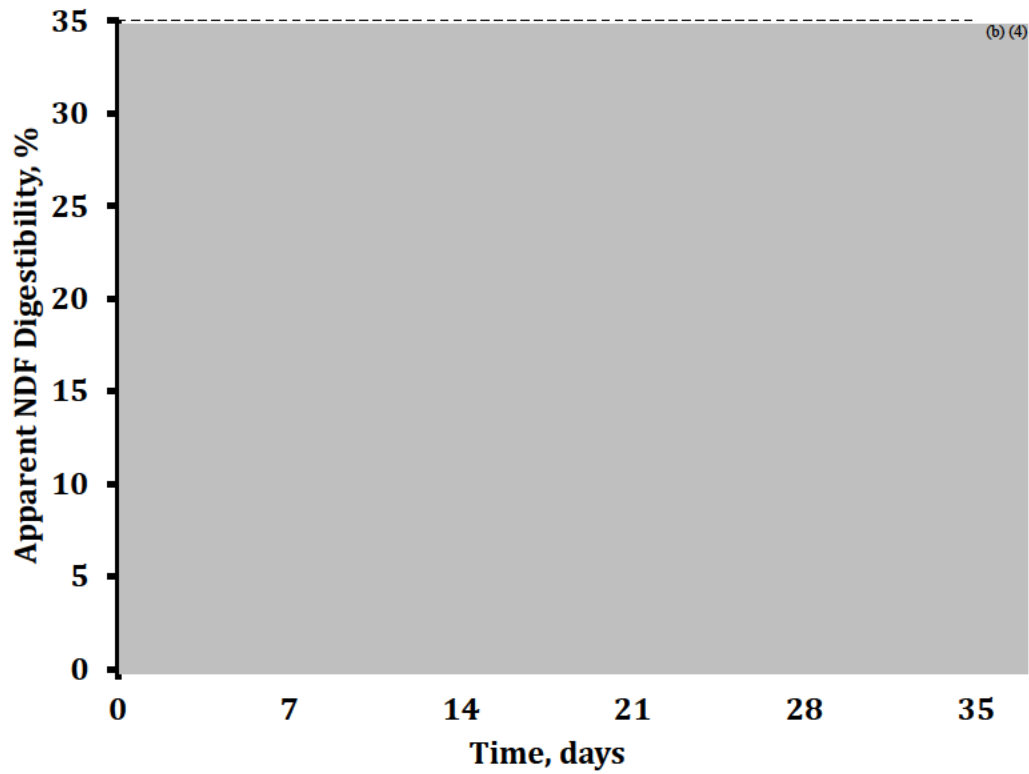
Figure III-3: Fecal NDF (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 5550) and treatment by time interaction (P = 0.5002). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).
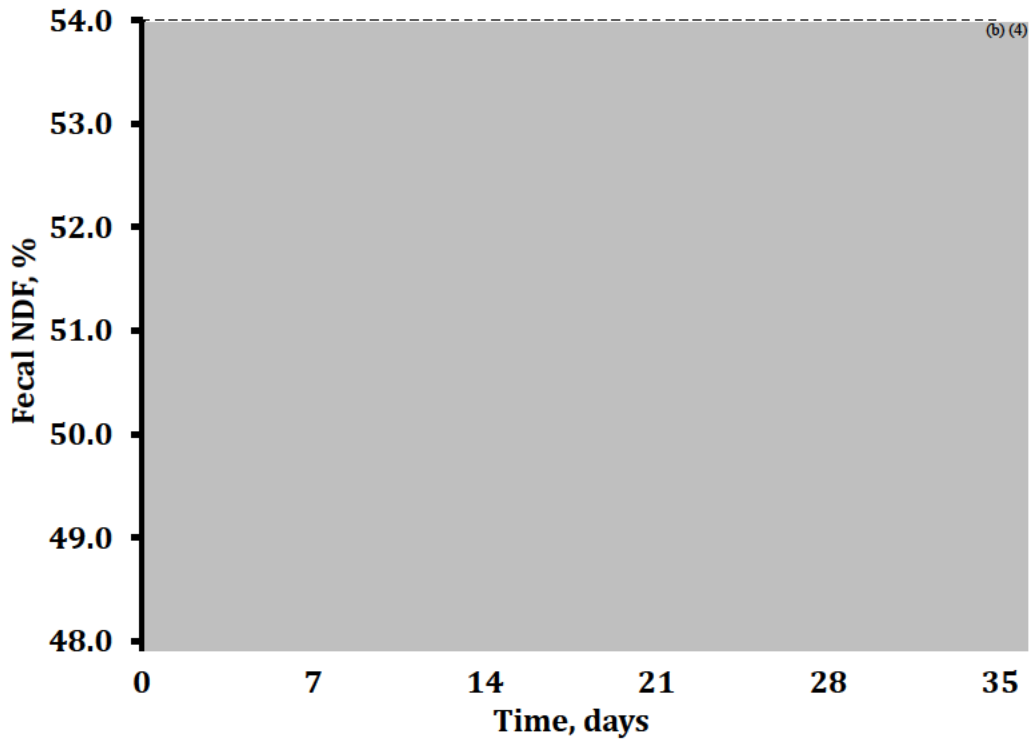
Figure III-4: Fecal protein (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 7876) and treatment by time interaction (P = 0.6687). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

Figure III-5: Fecal lignin (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 2080) and treatment by time interaction (P = 0.2597). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

Figure III-6: Apparent starch digestibility (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 0745) and treatment by time interaction (P = 0.6444). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

Figure III-7: Apparent NDF digestibility (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0. 2728) and treatment by time interaction (*P* = 0.6089). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).

Figure III-8: Apparent protein digestibility (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.6630$) and treatment by time interaction ($P = 0.2277$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).
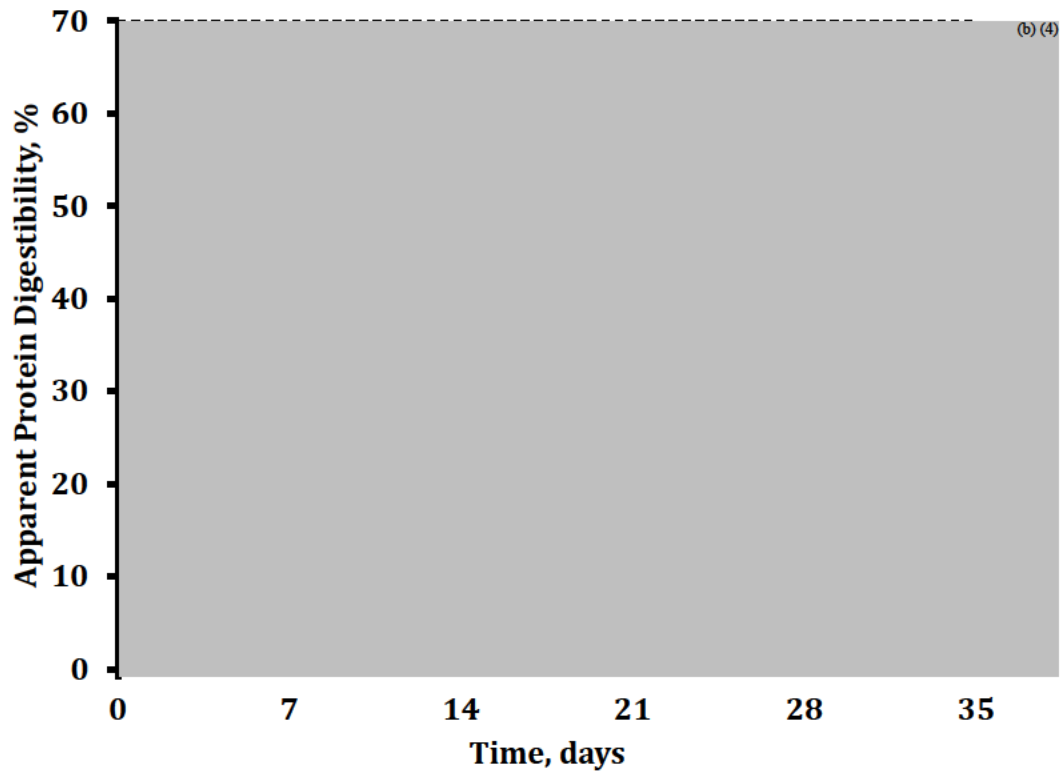
APPENDIX A (Materials and Methods)

Figure 0-1A: Schedule of events.

PRE-INTERVENTION PERIOD

Study Day	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
TMR Sampling									✓	
Fecal Sampling										✓
Digesta Sampling										
Cow Weight										✓
Feed Intake					✓	✓	✓	✓	✓	✓
Rumen pH										✓
Milk Yield					✓	✓	✓	✓	✓	✓
Milk Sampling										✓
Inocula ion										

INTERVENTION PERIOD

Study Days	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
TMR Sampling						✓						
Fecal Sampling							✓					
Digesta Sampling	✓	✓	✓		✓			✓			✓	
Cow Weight							✓					
Feed Intake	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Rumen pH	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Milk Yield	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Milk Sampling	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Inocula ion	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Study Days	Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24
TMR Sampling	✓							✓				
Fecal Sampling		✓							✓			
Digesta Sampling		✓			✓			✓			✓	
Cow Weight		✓							✓			
Feed Intake	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Rumen pH	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Milk Yield	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Milk Sampling	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Inocula ion	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Study Days	Day 25	Day 26	Day 27	Day 28	Day 29	Day 30	Day 31	Day 32
TMR Sampling			✓					
Fecal Sampling				✓				
Digesta Sampling		✓			✓			✓
Cow Weight				✓				
Feed Intake	✓	✓	✓	✓	✓	✓	✓	✓
Rumen pH	✓	✓	✓	✓	✓	✓	✓	✓
Milk Yield	✓	✓	✓	✓	✓	✓	✓	✓
Milk Sampling	✓	✓	✓	✓	✓	✓	✓	✓
Inocula ion	✓	✓	✓	✓	✓	✓	✓	✓

POST-INTERVENTION PERIOD

Study Days	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
TMR Sampling	✓				✓				✓	
Fecal Sampling		✓				✓				✓
Digesta Sampling	✓			✓			✓			✓
Cow Weight	✓					✓				✓
Feed Intake	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Rumen pH	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Milk Yield	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Milk Sampling	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Inocula ion										

Table 0-1A: Diet composition.

Ingredient	g/100g of DM
Alfalfa hay	7.79
Alfalfa green chop	5.98
Hay cubes	4.53
Corn silage	4.08
Wheat Silage	9.51
Almond Hulls	13.58
Citrus pulp	1.36
Wheat straw	0.89
Dry distillers grains	10.41
Steamed rolled corn	22.54
Canola	5.41
Cottonseed	5.33
Millrun	5.88
Salt	0.46
Molasses + Mineral and vitamin mix	2.26

Table 0-2A: Nutrient analysis of total mixed ration (TMR) offered to cows in both the control or microbial inoculation group during the Pre-Intervention (Pre), Intervention (I) and Post-Intervention (Post) periods.

Date	Study Day	Dry Matter (%)	Starch (% of DM)	NDF (% of DM)	Crude Protein (% of DM)	Lignin (% of DM)
1/26/16	Pre-9	66.7	21.0	28.9	17.6	6.1
2/2/16	I-6	64.2	22.5	25.4	17.7	5.0
2/9/16	I-13	66.5	17.9	28.7	17.2	5.5
2/16/16	I-20	66.8	20.6	26.7	17.2	5.1
2/23/16	I-27	67.8	21.6	26.8	17.5	5.4
2/29/16	Post-1	68.2	22.1	25.4	17.2	5.0
3/4/16	Post-5	69.3	21.2	26.7	17.1	5.3
3/8/16	Post-9	65.7	19.8	28.8	17.6	5.8

APPENDIX B (Section I)

Results

This analysis (n = 8) excluded cow IDs 3, 7, 8, 14 and 15. Treatment least square means, fixed effects and covariance parameters were estimated using the models described in Section I and are reported in Table I-1B and Figures I-1B to I-13B. Milk fat percentage was still numerically higher for Inoculated, but was neither significant nor tended to be significant. A treatment by time interaction was observed for milk yield ($P = 0.0271$, Figure I-2B) and milk protein yield ($P = 0.0274$, Figure I-8B). Milk and protein yields for Inoculated were higher on week 2 and lower on week 5 compared to the control group.

Table I-1B: Dry matter intake, milk production and composition, BW gain and rumen pH least square means (± SEM) of cows assigned to Control and Inoculated.

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Outcome	Treatment		Fixed Effect[1]			
	Control	Inoculated	Cov	Tx	Week	Tx* Week
						(b) (4)
DMI, kg	32.4 ± 1.1	32.0 ± 1.0				
Milk yield, kg	32.7 ± 0.8	33.1 ± 0.7				
FCM, kg	34.5 ± 1.3	35.4 ± 1.2				
ECM, kg	33.8 ± 1.2	34.9 ± 1.1				
Milk components, %						
Crude Protein	3.04 ± 0.11	3.22 ± 0.10				
Fat	3.77 ± 0.10	4.00 ± 0.10				
Lactose	4.76 ± 0.06	4.72 ± 0.06				
Milk components yield, kg						
Crude Protein	1.00 ± 0.03	1.05 ± 0.03				
Fat	1.24 ± 0.05	1.31 ± 0.05				
MUN, mg/dL	7.00 ± 0.55	7.46 ± 0.50				
FCM/DMI	1.11 ± 0.05	1.12 ± 0.04				
BW gain, kg/day	1.68 ± 0.38	1.33 ± 0.32				
Rumen pH	6.16 ± 0.11	6.04 ± 0.10				

[1]Cov= covariate effect, Tx = treatment effect, Day = day effect; Tx*Day = treatment by day interaction.

Figure I-1B: Dry matter intake (kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.8273) and treatment by time interaction (P = 0.9269). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).
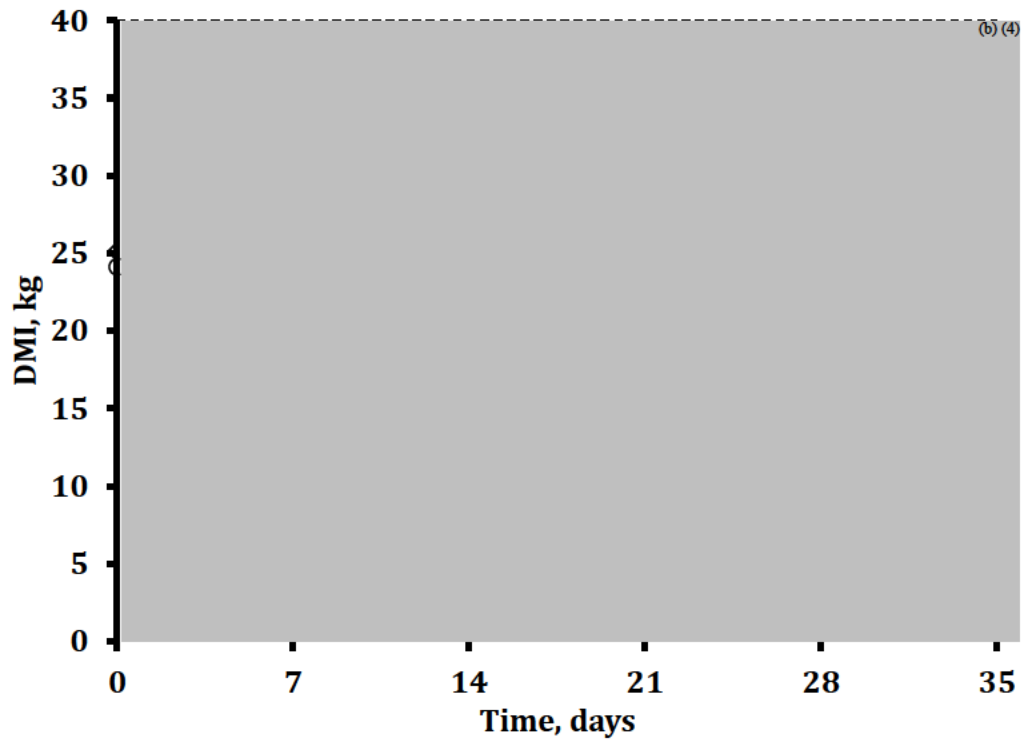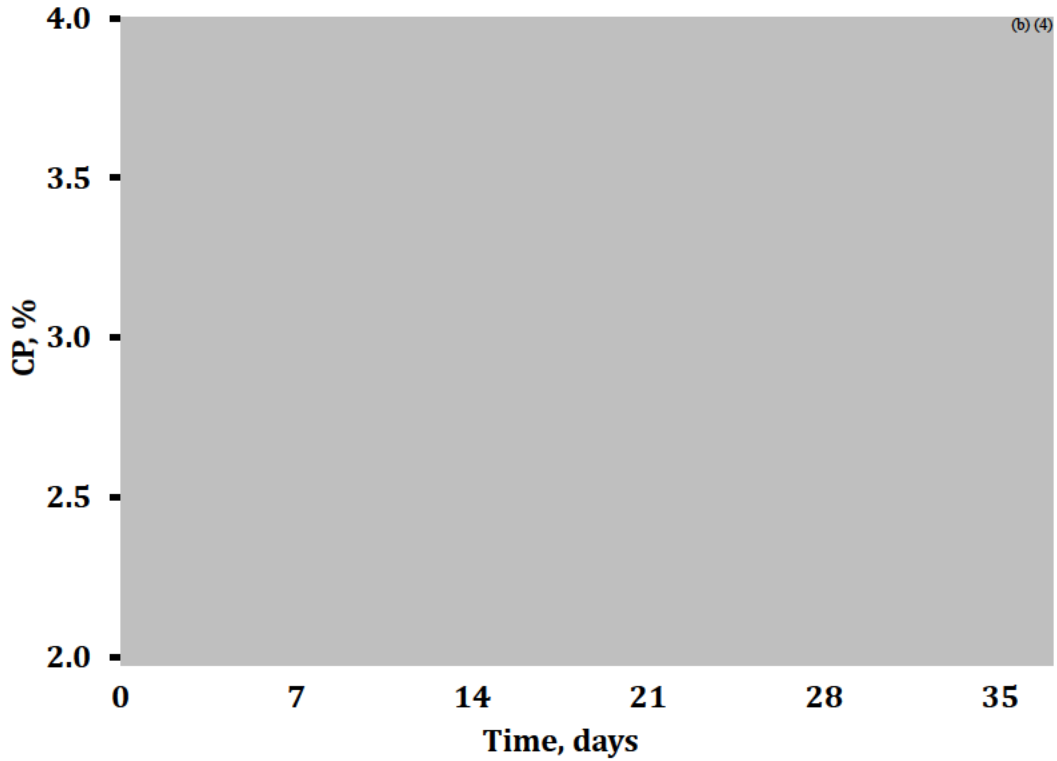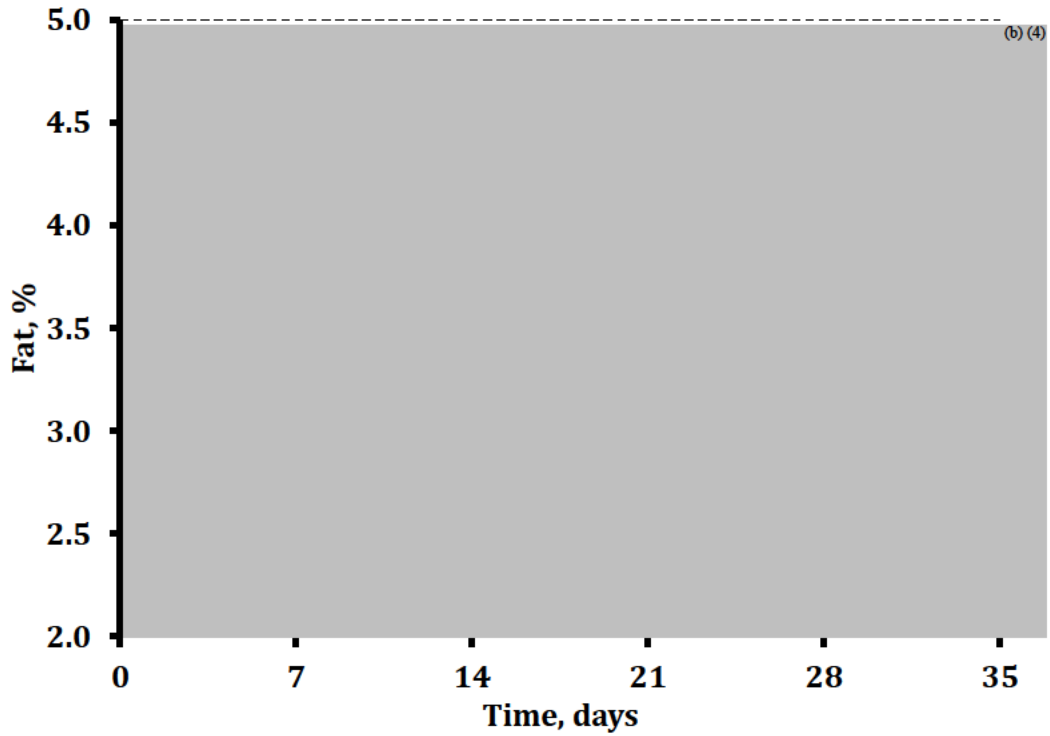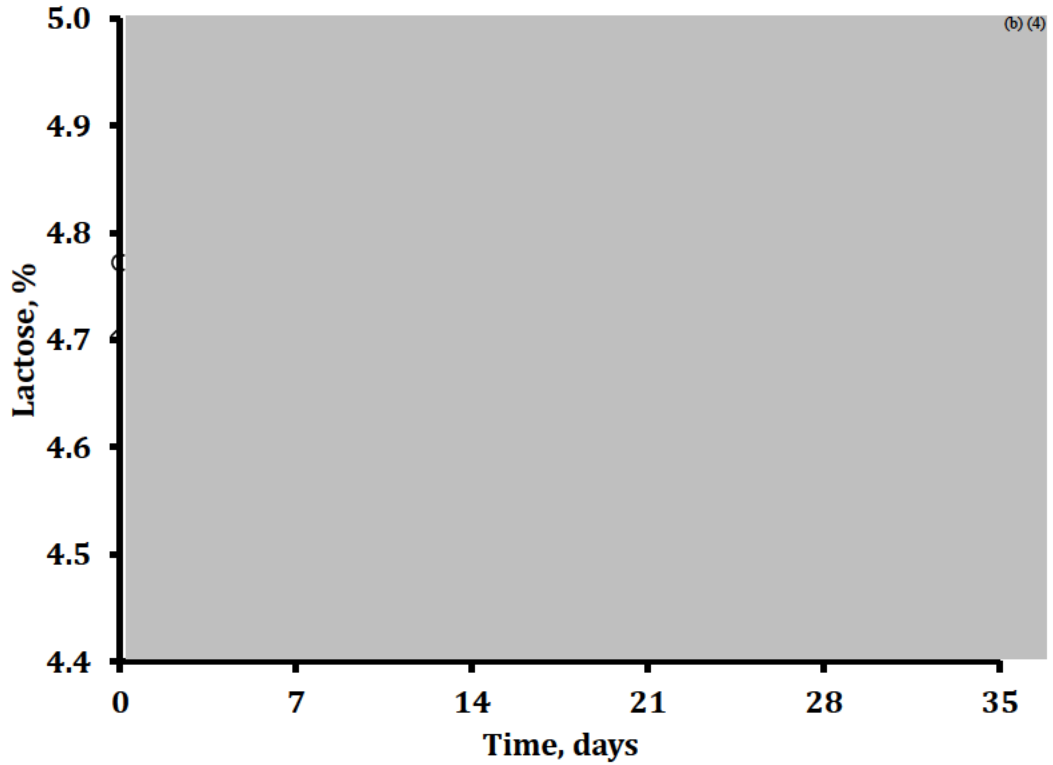
Figure I-2B: Milk yield (kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.7282$) and treatment by time interaction ($P = 0.0271$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).
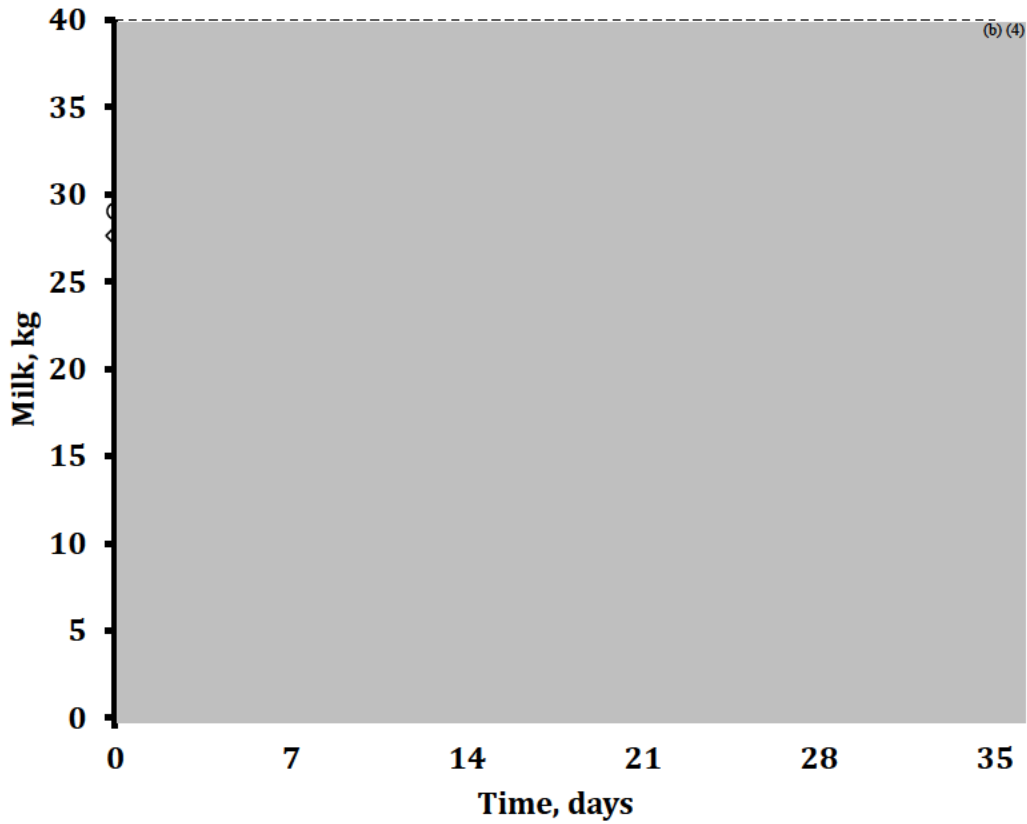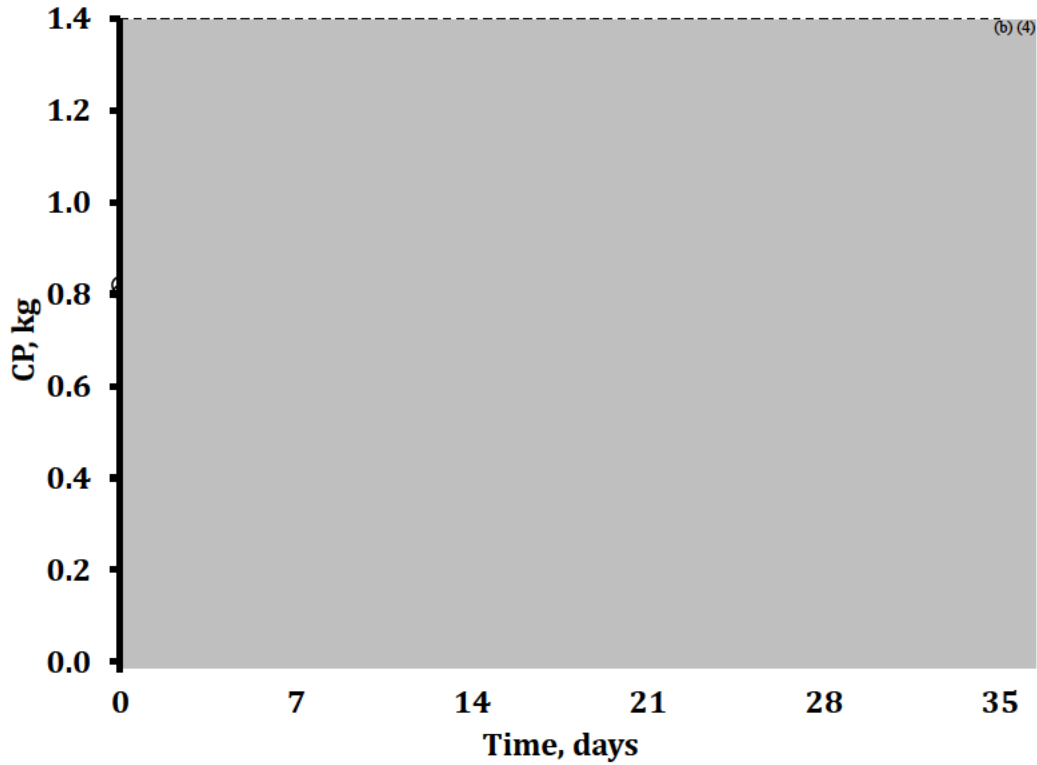
Figure I-3B: Fat corrected milk yield (FCM, kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.6267$) and treatment by time interaction ($P = 0.0948$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).
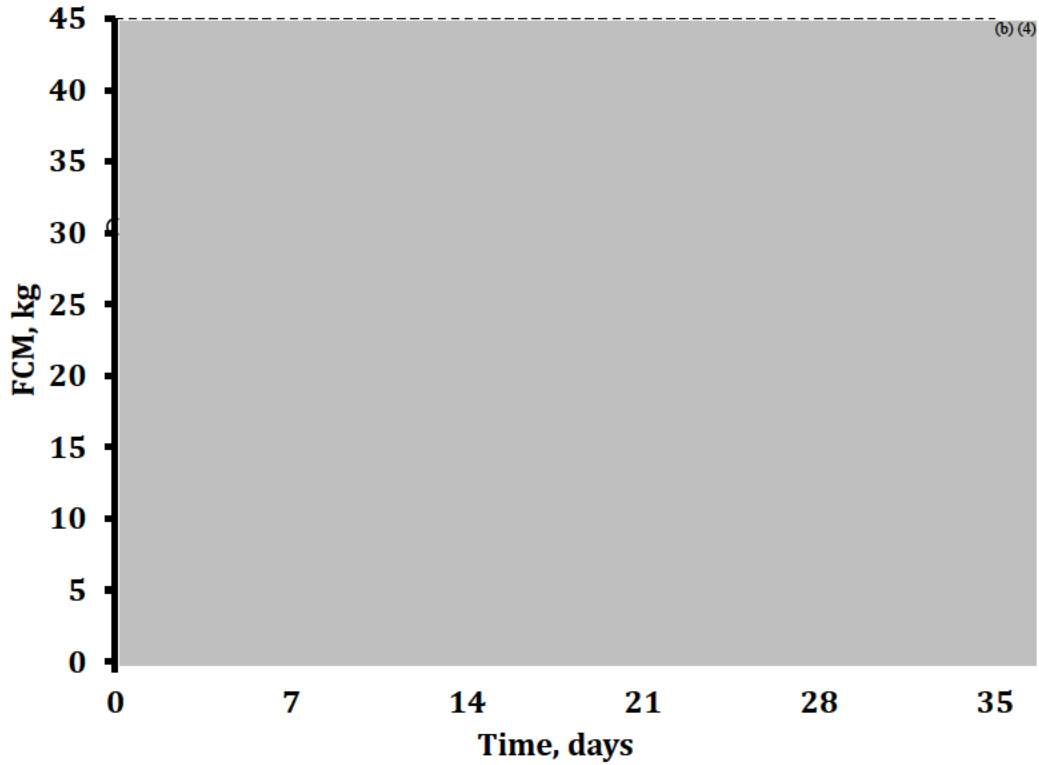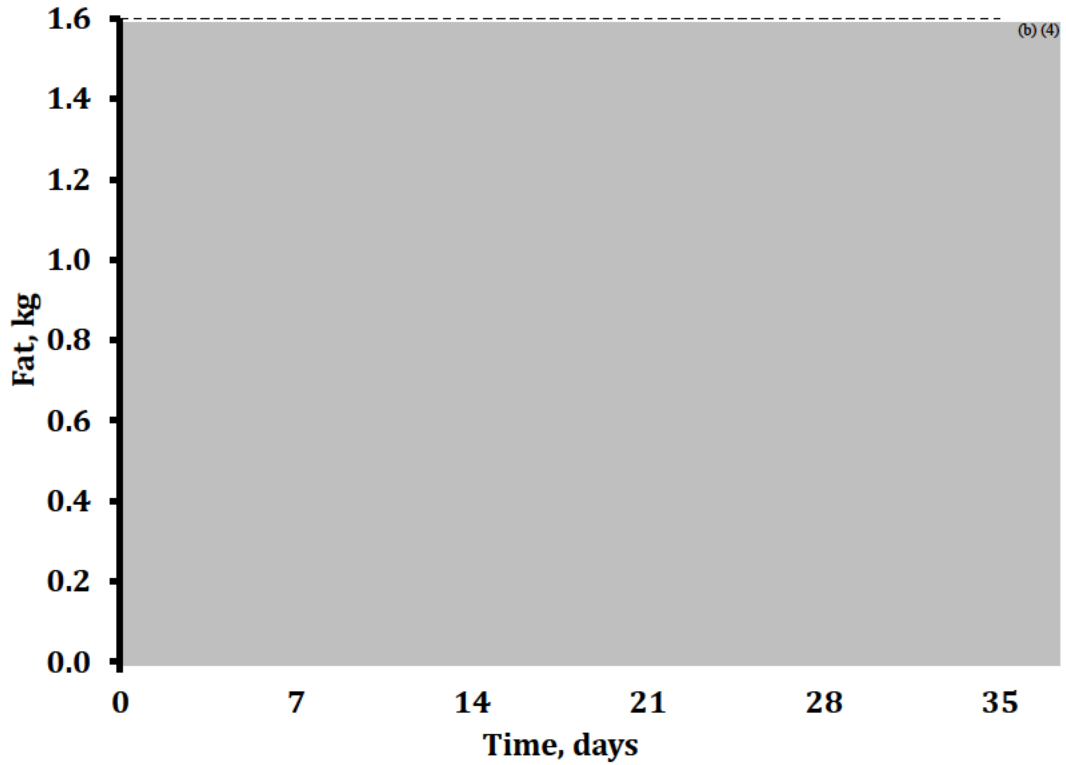
Figure I-4B: Energy corrected milk yield (ECM, kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.5339$) and treatment by time interaction ($P = 0.0670$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).
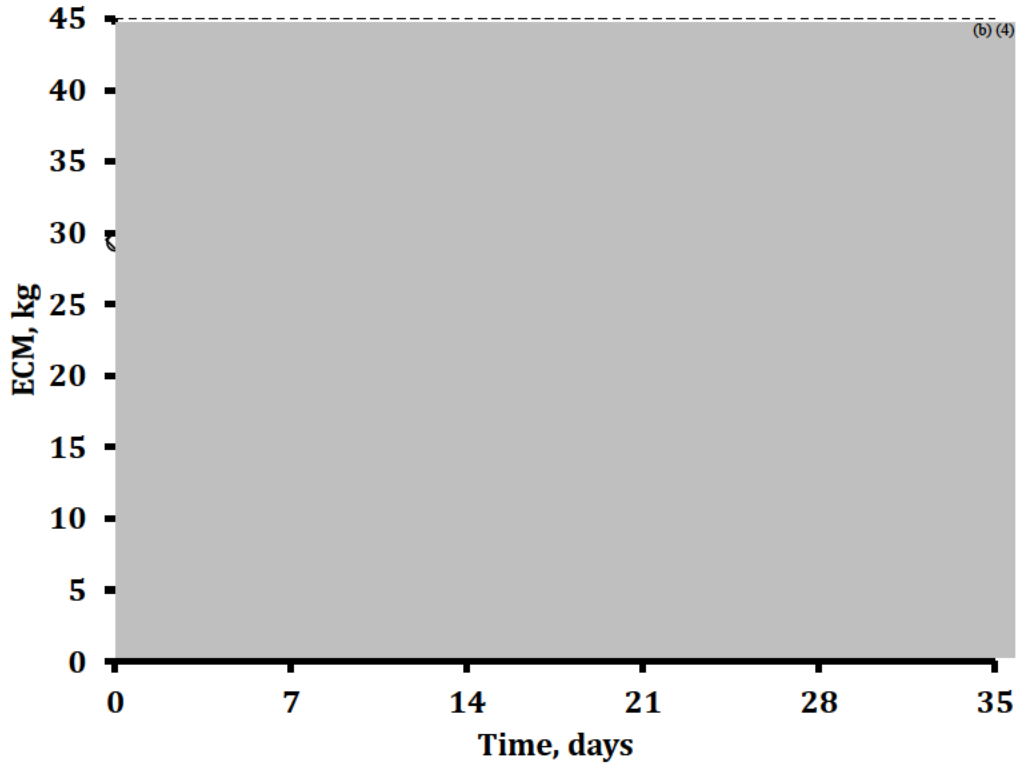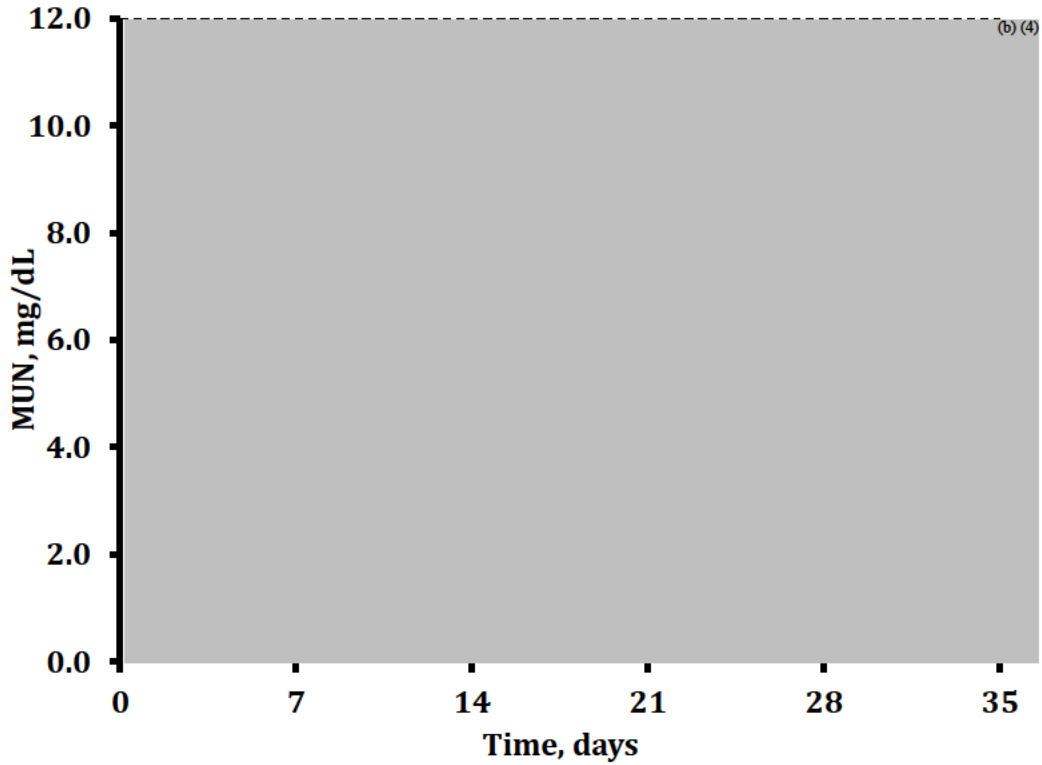
Figure I-5B: Milk crude protein (CP, %) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.2352$) and treatment by time interaction ($P = 0.0971$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).

Figure I-6B: Milk fat (%) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1346$) and treatment by time interaction ($P = 0.4820$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).
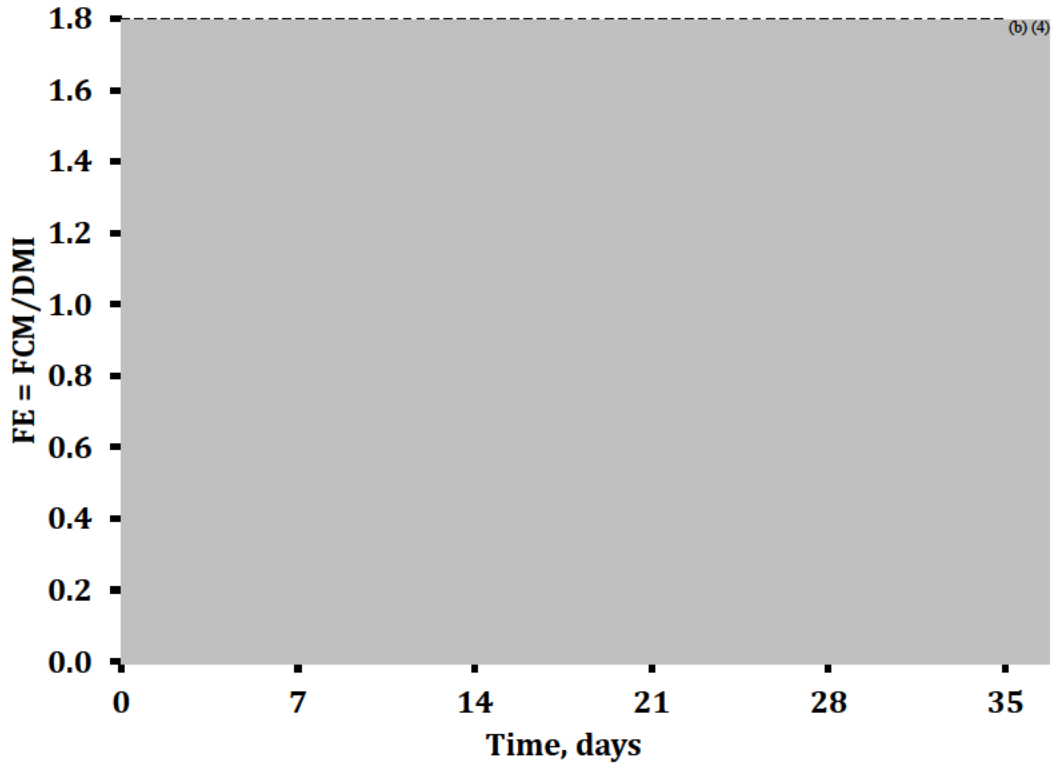
Figure I-7B: Milk lactose (%) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.6333) and treatment by time interaction (P = 0.3795). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).
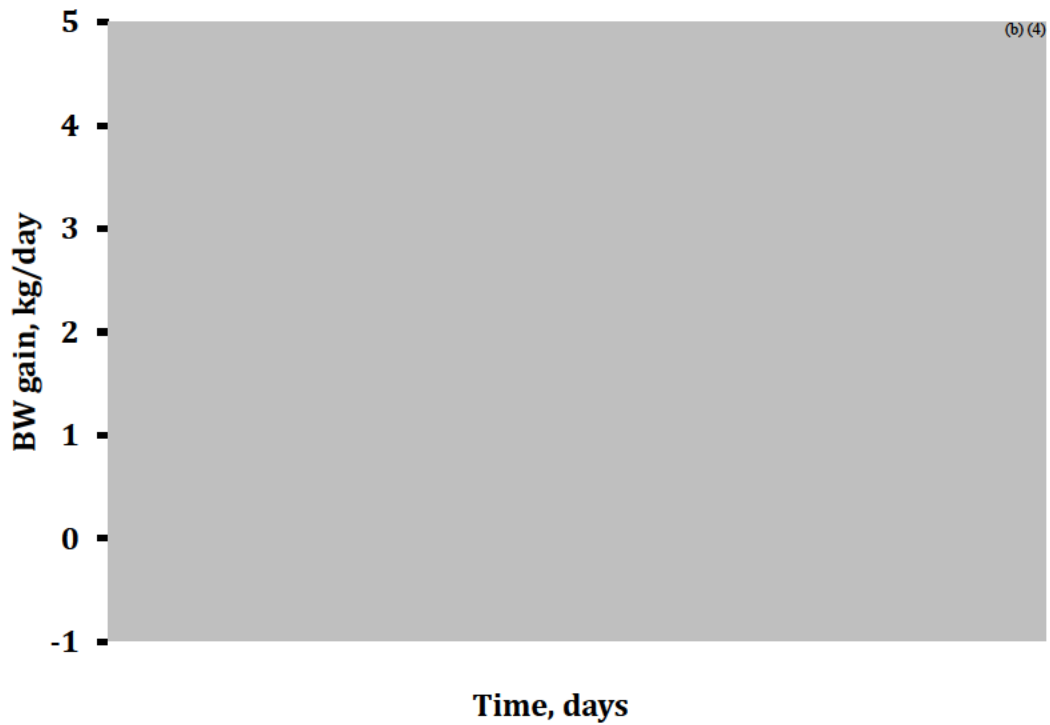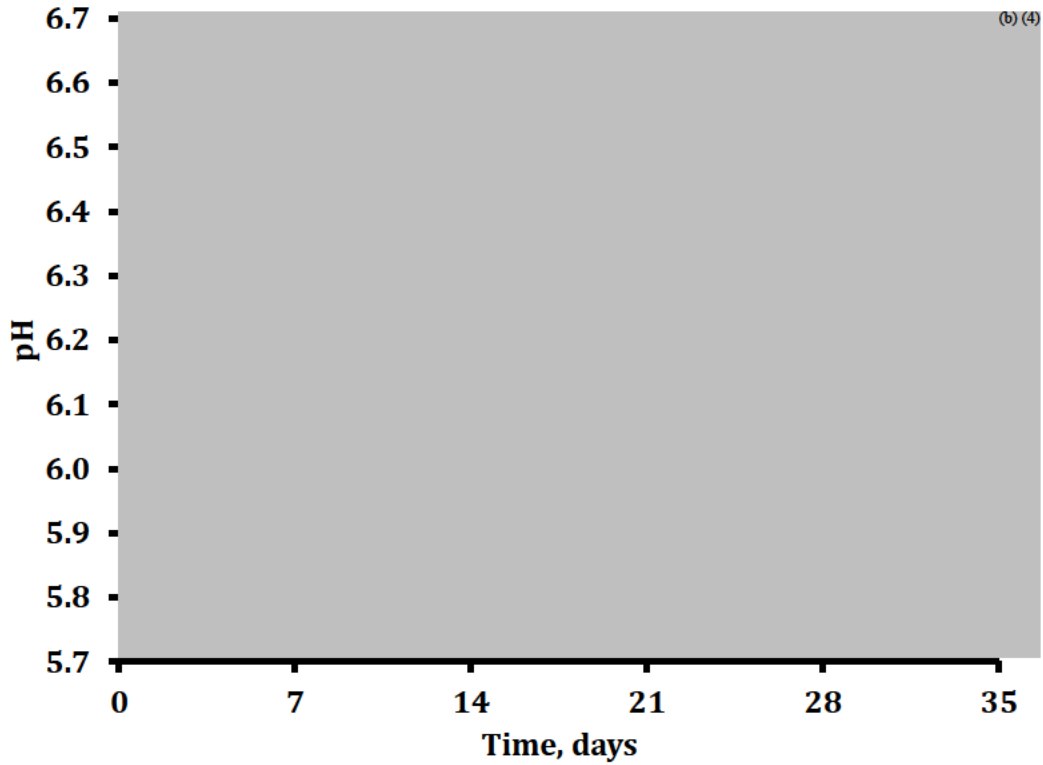
Figure I-8B: Milk crude protein yield (CP, kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.3111) and treatment by time interaction (P = 0.0274). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

Figure I-9B: Milk fat yield (kg) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.3727$) and treatment by time interaction ($P = 0.2287$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).

Figure I-10B: Milk urea nitrogen (MUN, mg/dL) daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.5513$) and treatment by time interaction ($P = 0.7861$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$).

Figure I-11B: Feed efficiency (FCM/DMI) means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.8765) and treatment by time interaction (P = 0.0810). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

Figure I-12B: BW gain (kg/day) weekly least square means± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 4919) and treatment by time interaction (P = 0.9799). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

Figure I-13B: Rumen pH daily means (no fill) and weekly least square means (solid fill) ± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.4334) and treatment by time interaction (P = 0.3331). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

APPENDIX C (Section II)

This analysis (n = 8) excluded cow IDs 3, 7, 8, 14 and 15. Treatment least square means, fixed effects and covariance parameters were estimated using the models described in Section II.

Figure II-1C: Dry matter intake (kg) daily means (no fill), covariate adjusted weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.2201$) and treatment by time interaction ($P = 0.1910$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.
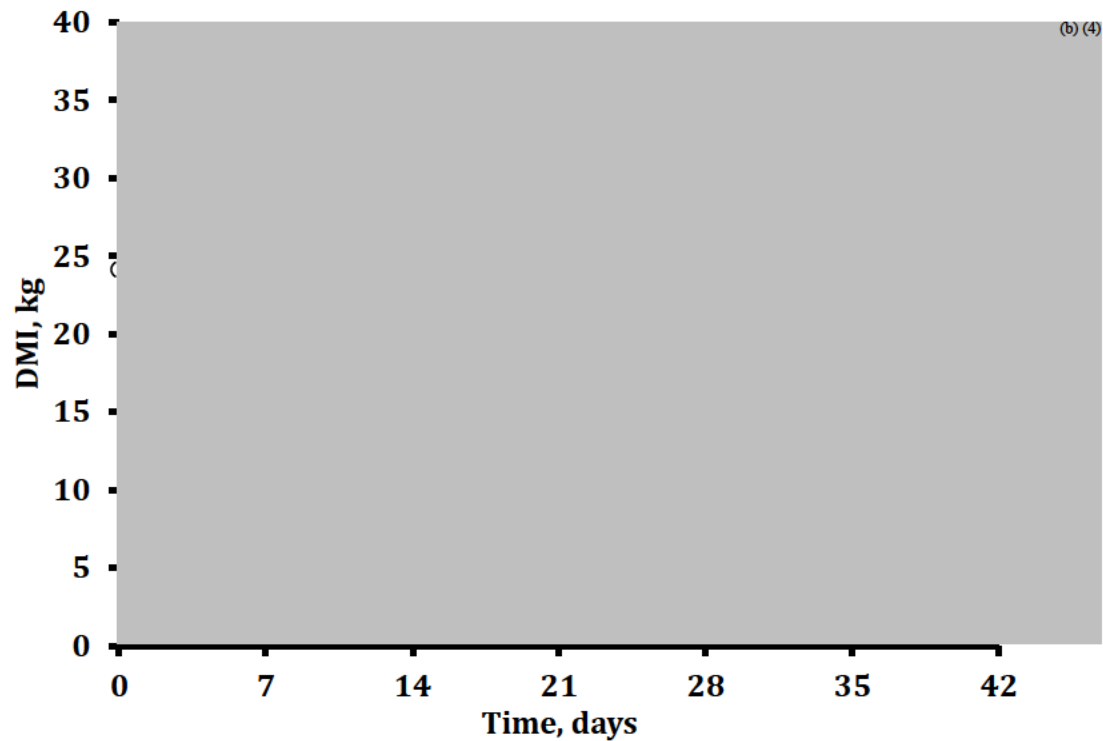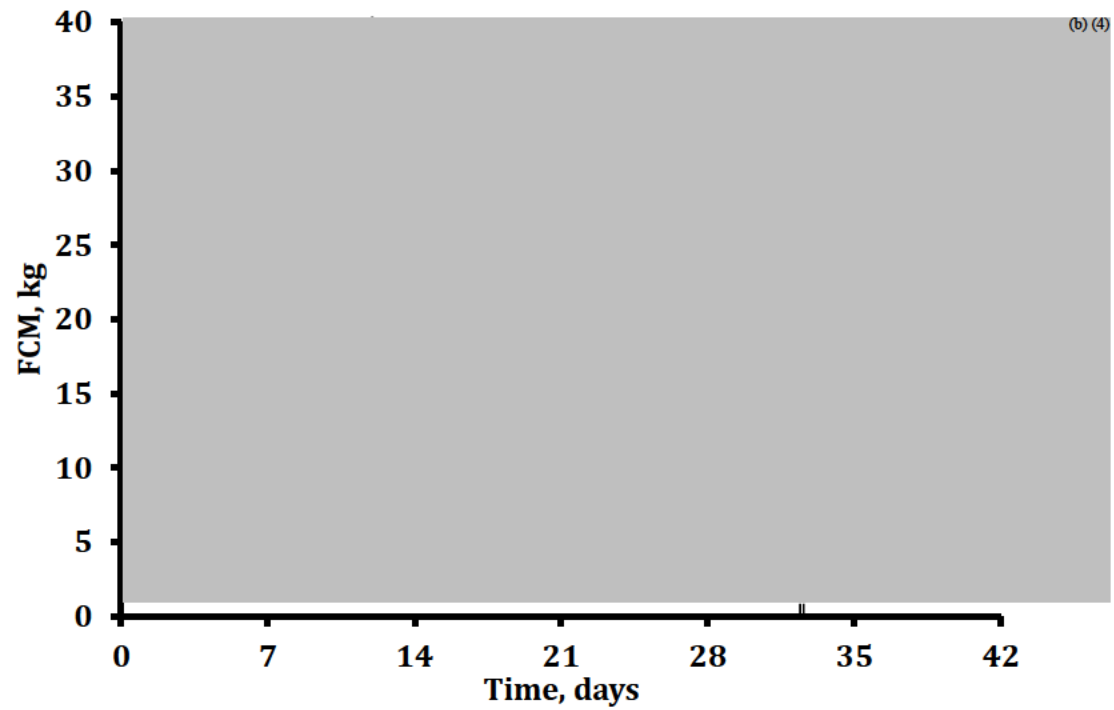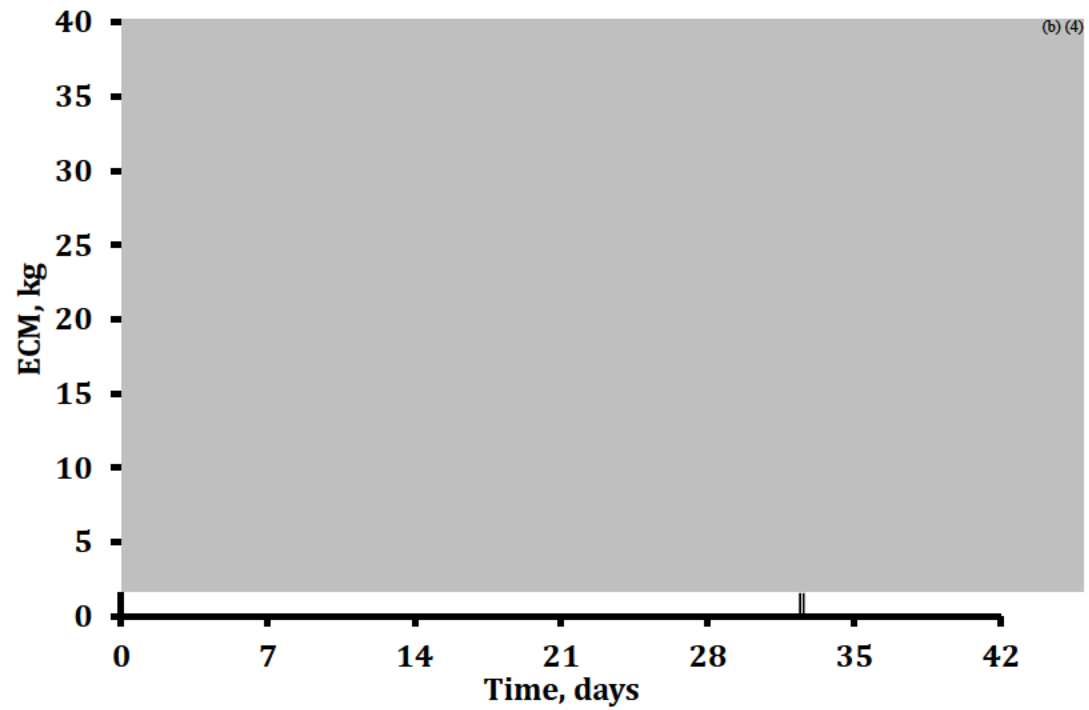
Figure II-2C: Milk yield (kg) daily means (no fill), covariate adjusted weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 0791) and treatment by time interaction (P = 0.0025). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01). The vertical line represents the end of the feeding trial.
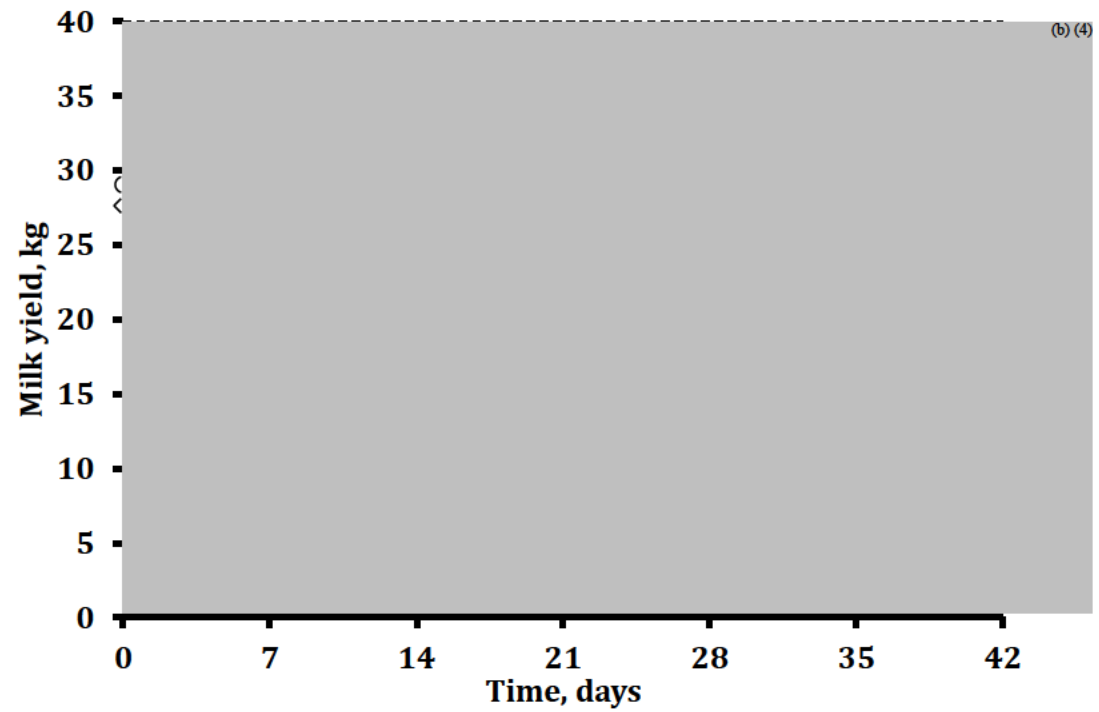
Figure II-3C: Fat corrected milk yield (FCM, kg) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1883$) and treatment by time interaction ($P = 0.0026$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.
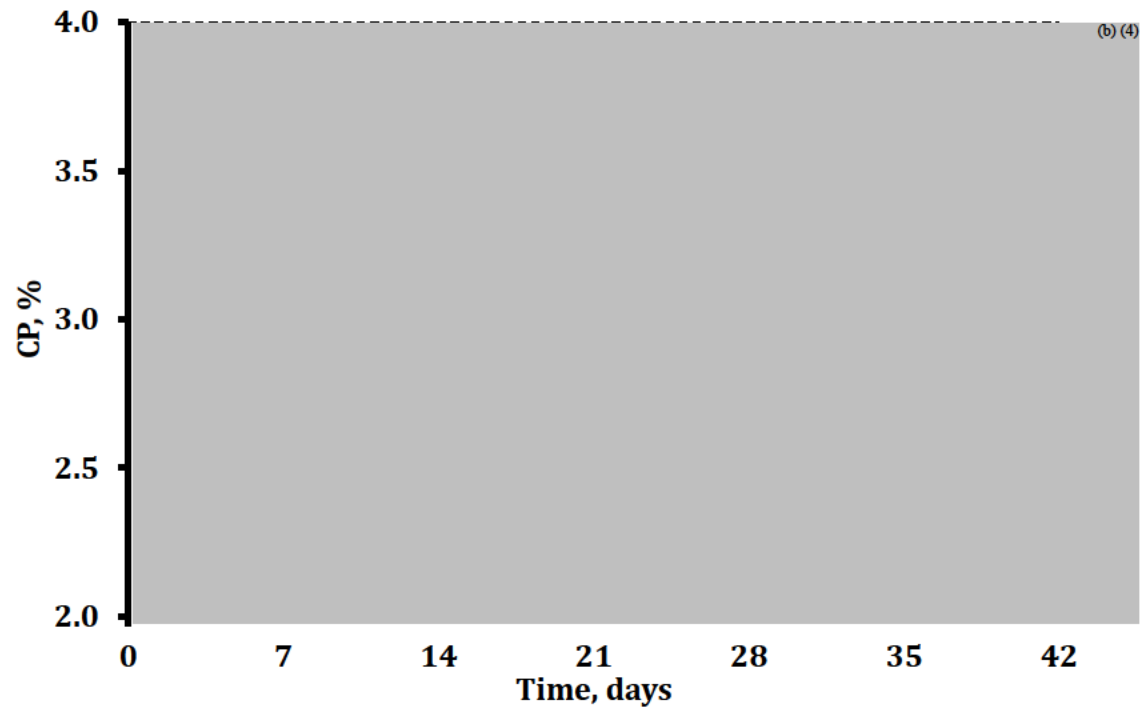
Figure II-4C: Energy corrected milk yield (ECM, kg) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1669$) and treatment by time interaction ($P = 0.0019$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.
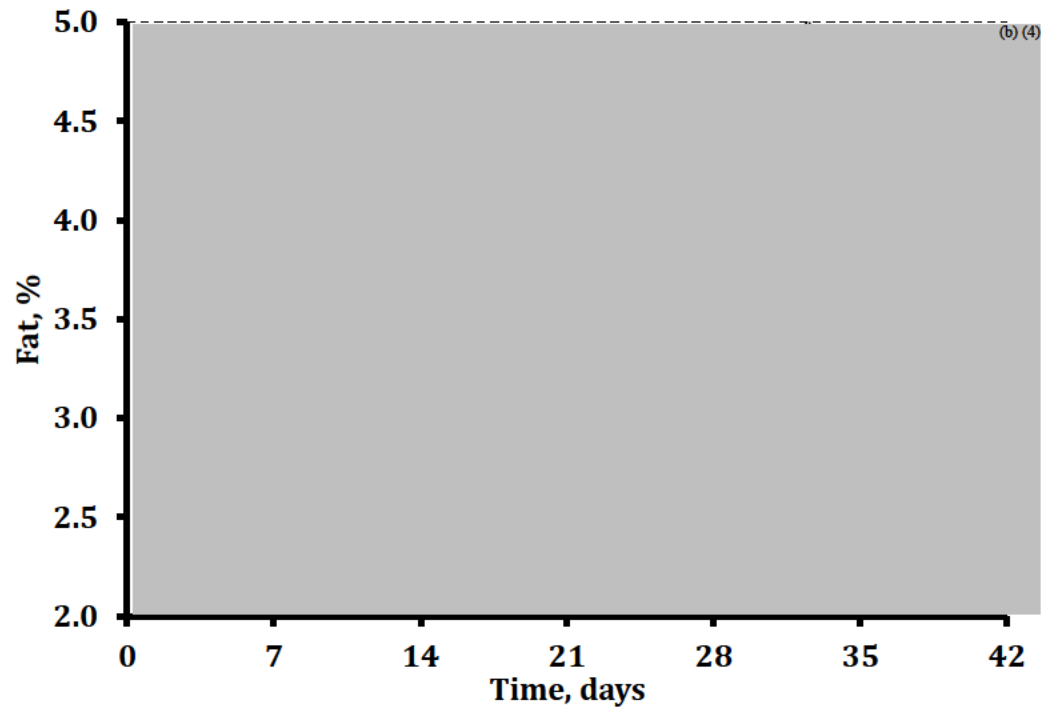
Figure II-5C: Milk crude protein (CP, %) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1553$) and treatment by time interaction ($P = 0.3125$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.
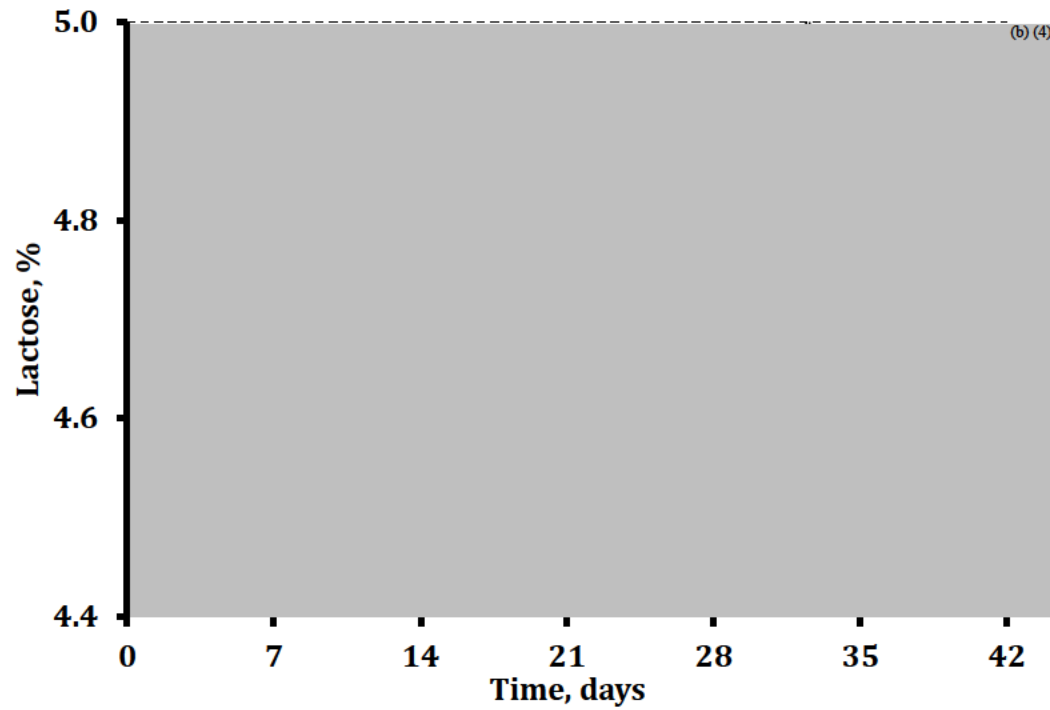
Figure II-6C: Milk fat (%) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.0991$) and treatment by time interaction ($P = 0.2677$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.
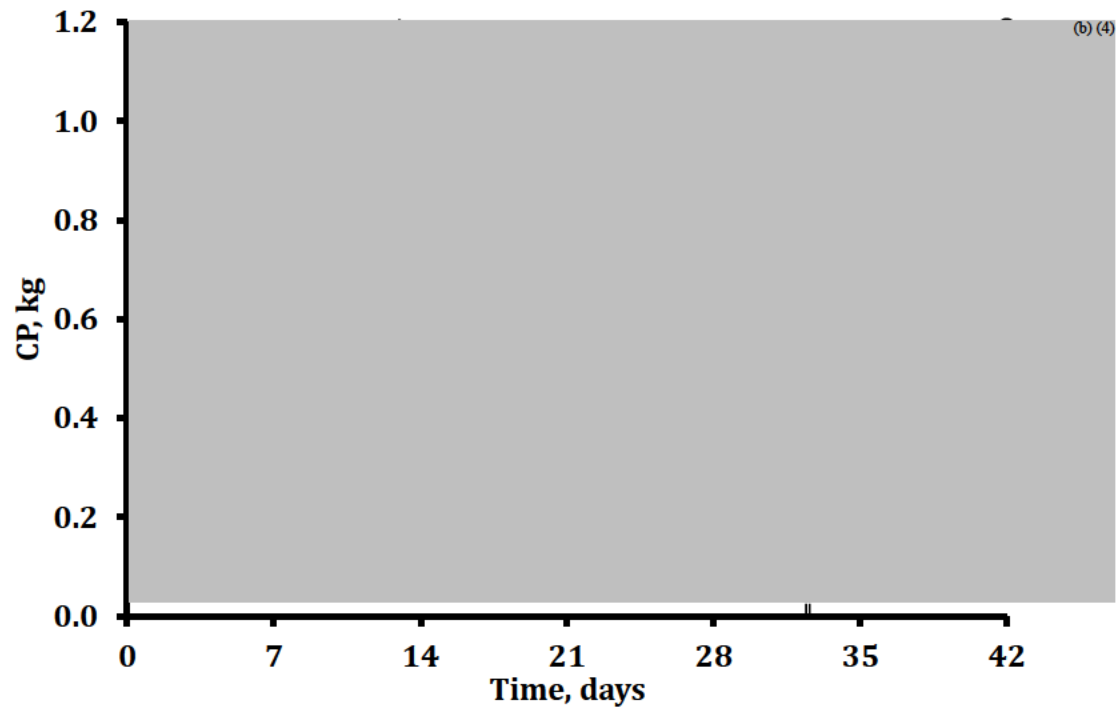
Figure II-7C: Milk lactose (%) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.3787) and treatment by time interaction (P = 0.5016). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01). The vertical line represents the end of the feeding trial.

Figure II-8C: Milk crude protein yield (CP, kg) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1183) and treatment by time interaction (P = 0.0012). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01). The vertical line represents the end of the feeding trial.
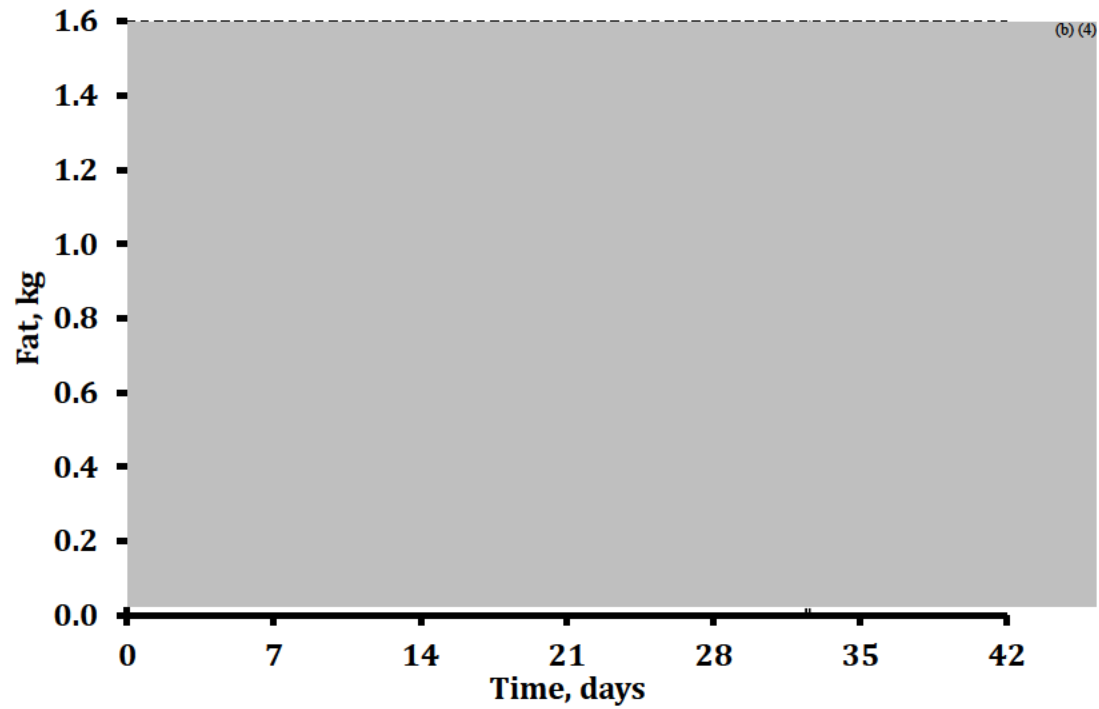
Figure II-9C: Milk fat yield (kg) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.1818) and treatment by time interaction (P = 0.0880). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01). The vertical line represents the end of the feeding trial.
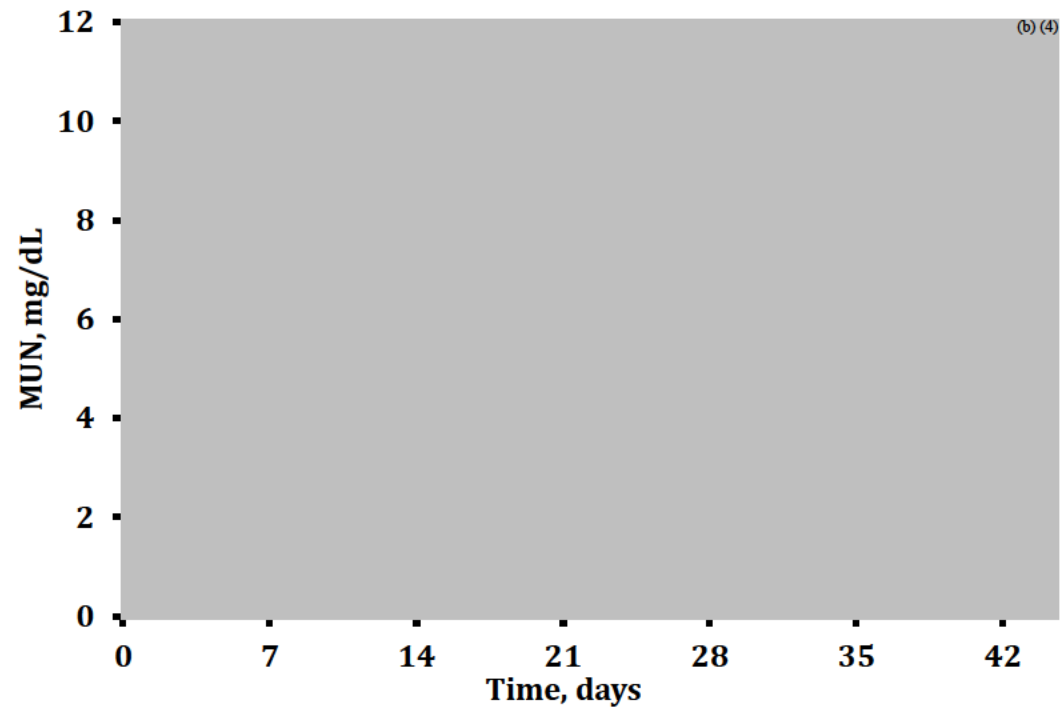
Figure II-10C: Milk urea nitrogen (MUN, mg/dL) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1222$) and treatment by time interaction ($P = 0.3440$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.
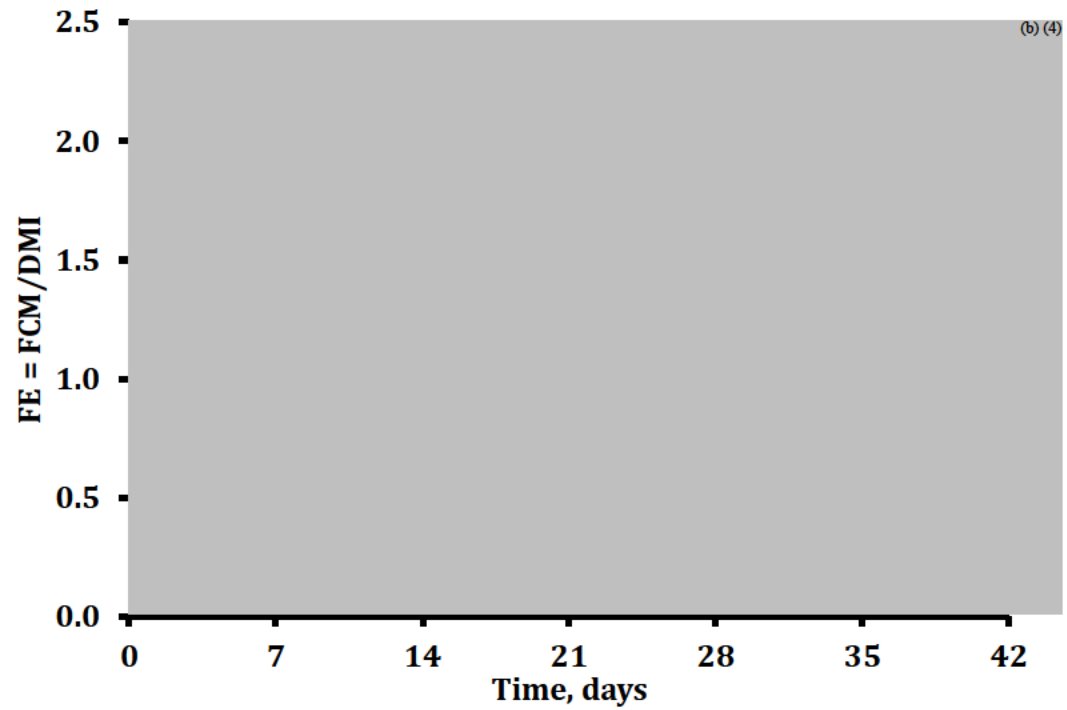
Figure II-11C: Feed efficiency (FCM/DMI) daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.2835$) and treatment by time interaction ($P = 0.0671$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.
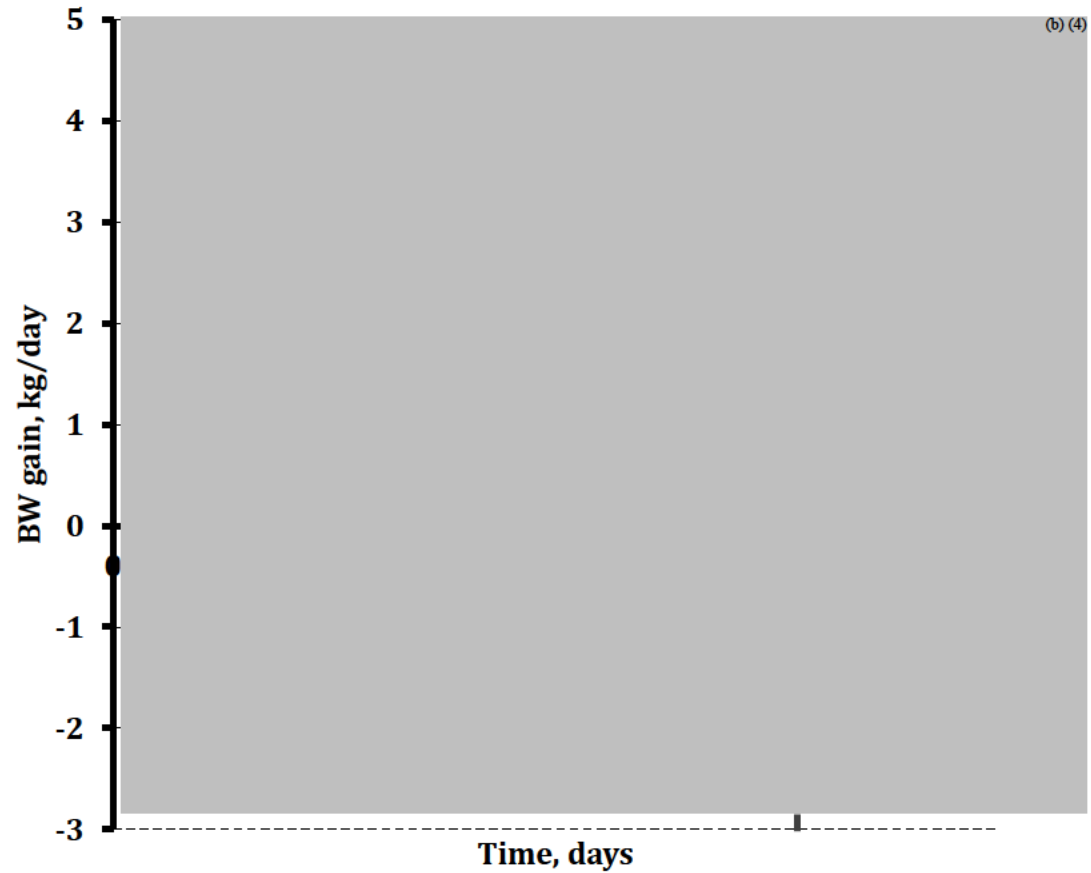
Figure II-12C: BW gain (kg/day) weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) weekly least square means± SEM of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.2838$) and treatment by time interaction ($P = 0.3335$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.

Figure II-13C: Rumen pH daily means (no fill), weekly Intervention least square means (solid black fill) ± SEM and Post-Intervention trial means (solid grey fill) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment ($P = 0.1600$) and treatment by time interaction ($P = 0.0741$). Treatment effect within week was established when a significant treatment by time interaction was observed (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$). The vertical line represents the end of the feeding trial.
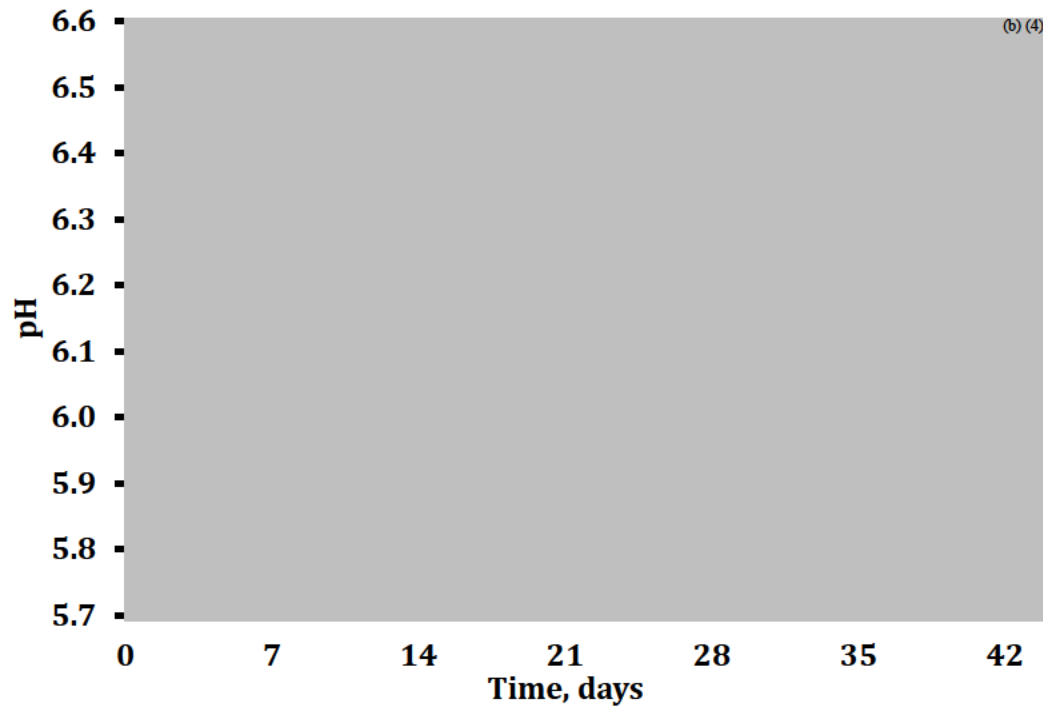
APPENDIX D (Section III)

Results

This analysis (n = 4) excluded 4 fecal pools that included cows IDs 3, 7, 8, 14 and 15. Treatment least square means, fixed effects and covariance parameters were estimated using the models described in Section III and are reported in Table III-1D and Figures III-1D to III-13D. No significant treatment by week interaction was observed on any of the outcomes measured. Apparent protein digestibility was higher for Inoculated vs Control (P = 0.0143).

Table III-1D: Fecal matter concentration and digestibility least square means of cows assigned either to Control or Inoculated.

| Outcome | Treatment | | | Fixed Effects[1] | | | |
	Control	Inoculated	SEM	Cov	Tx	Week	Tx*Week
Fecal matter, %							(b) (4)
DM	15.7	15.8	1.0				
Starch	6.7	7.2	0.8				
NDF	50.5	51.4	0.1				
Protein	19.5	19.0	0.3				
Lignin	10.6	10.8	0.3				
Digestibility							
Starch	87.5	86.7	1.4				
NDF	17.5	19.2	3.3				
Protein	50.8	53.4	0.1				

[1]Cov= covariate effect, Tx = treatment effect, Day = day effect; Tx*Day = treatment by day interaction.

Figure III-1D: Fecal DM (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0. 9640) and treatment by time interaction (*P* = 0.2329). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).
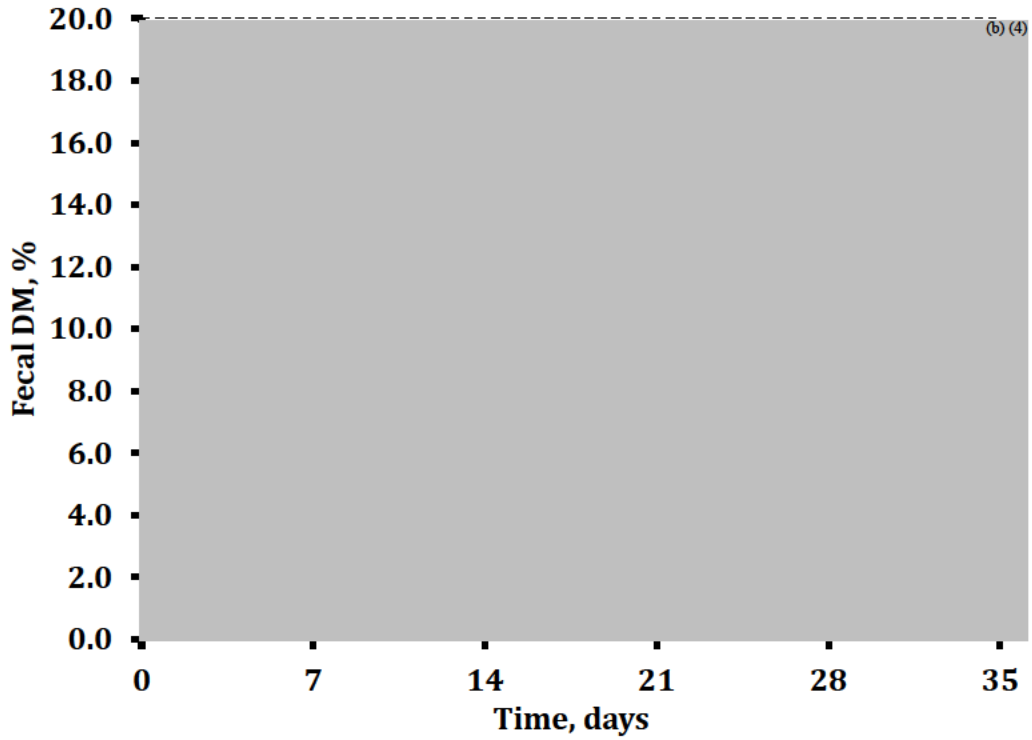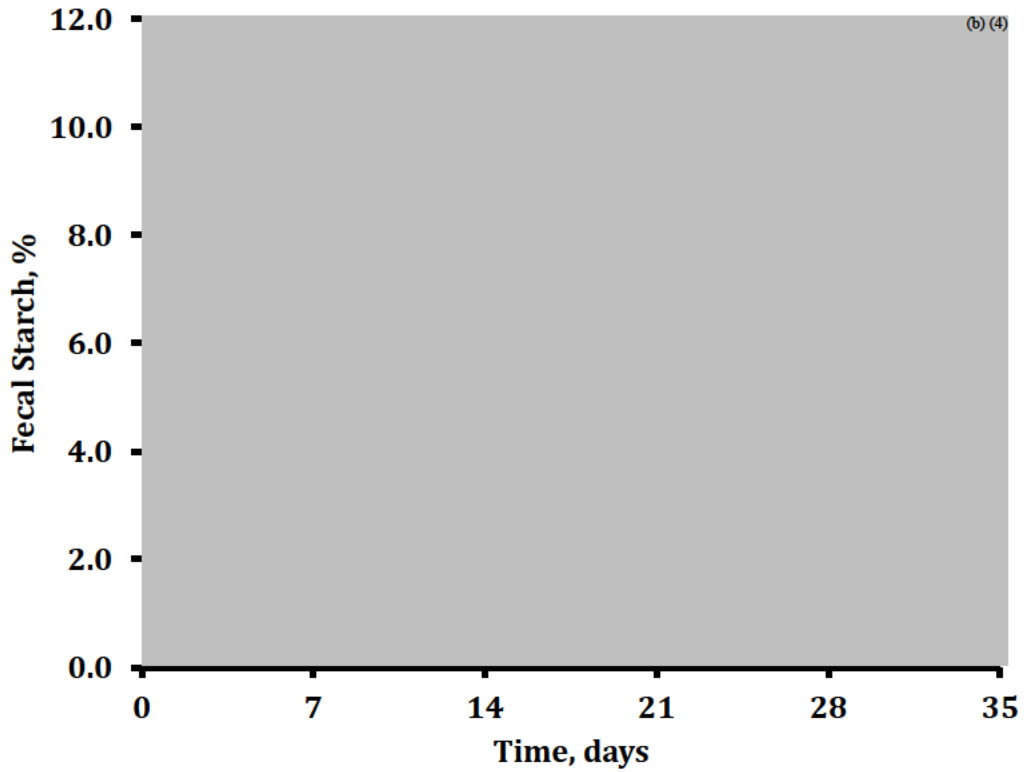
Figure III-2D: Fecal Starch (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 7626) and treatment by time interaction (P = 0.1666). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).

Figure III-3D: Fecal NDF (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 1121) and treatment by time interaction (P = 0.5054). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).
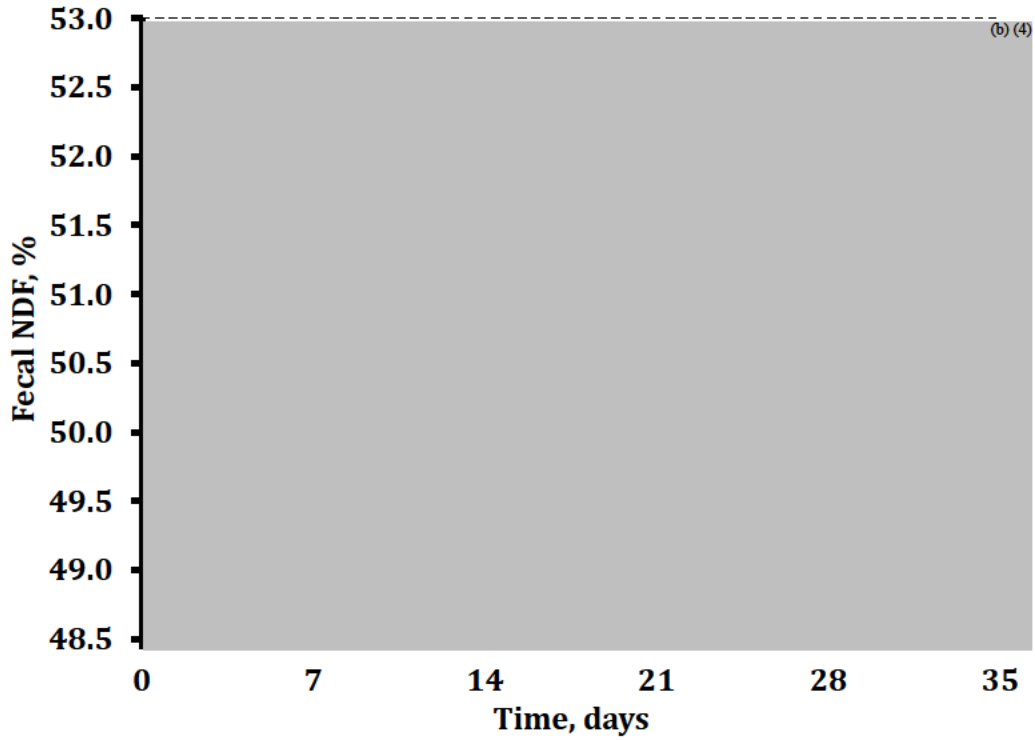
Figure III-4D: Fecal protein (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 4977) and treatment by time interaction (P = 0.8478). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).
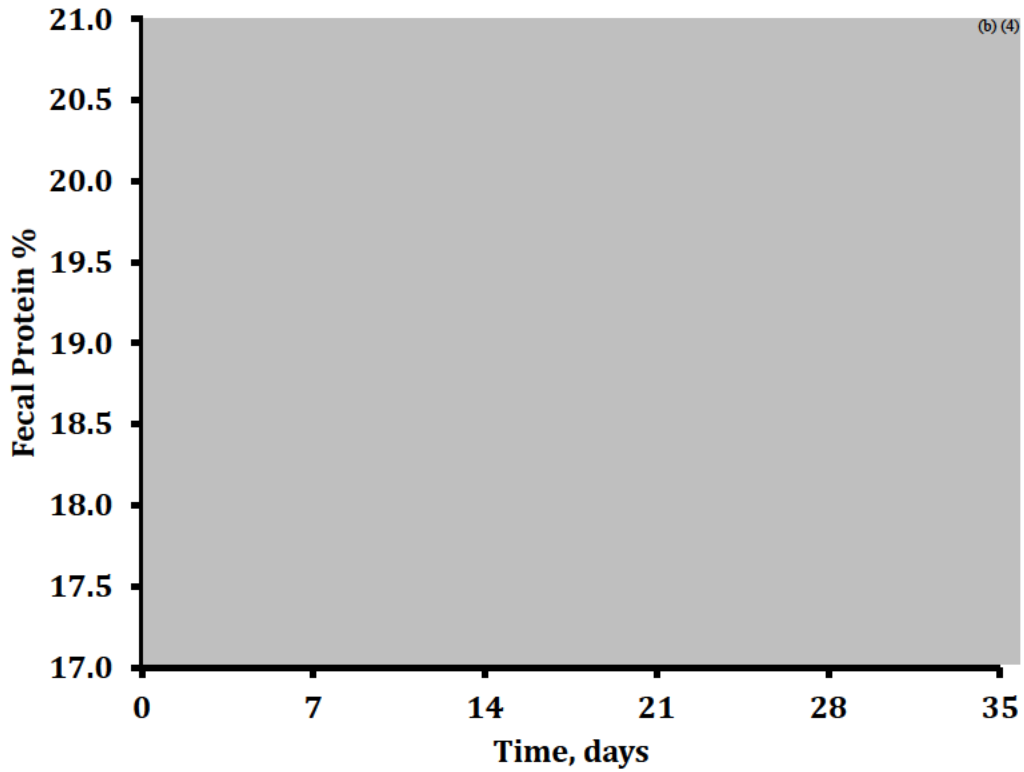
Figure III-5D: Fecal lignin (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0. 6845) and treatment by time interaction (P = 0.2826). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).
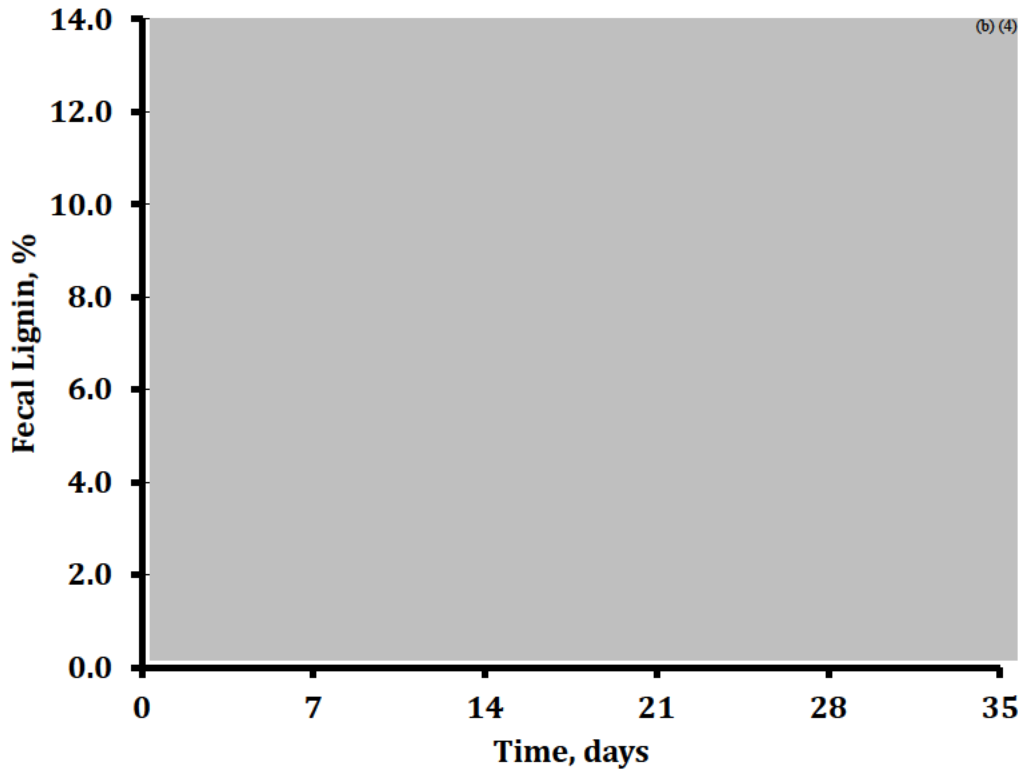
Figure III-6D: Apparent starch digestibility (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0.8063) and treatment by time interaction (*P* = 0.6958). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).
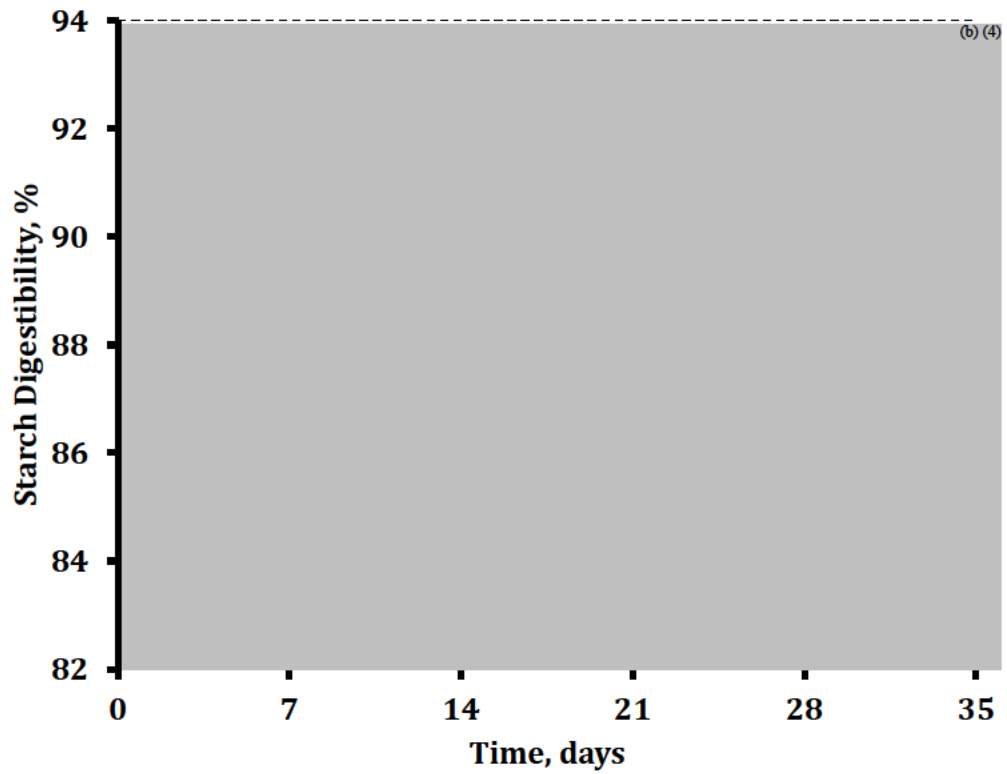
Figure III-7D: Apparent NDF digestibility (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (P = 0.7936) and treatment by time interaction (P = 0.4751). Treatment effect within week was established when a significant treatment by time interaction was observed (*P < 0.10, **P < 0.05, ***P < 0.01).
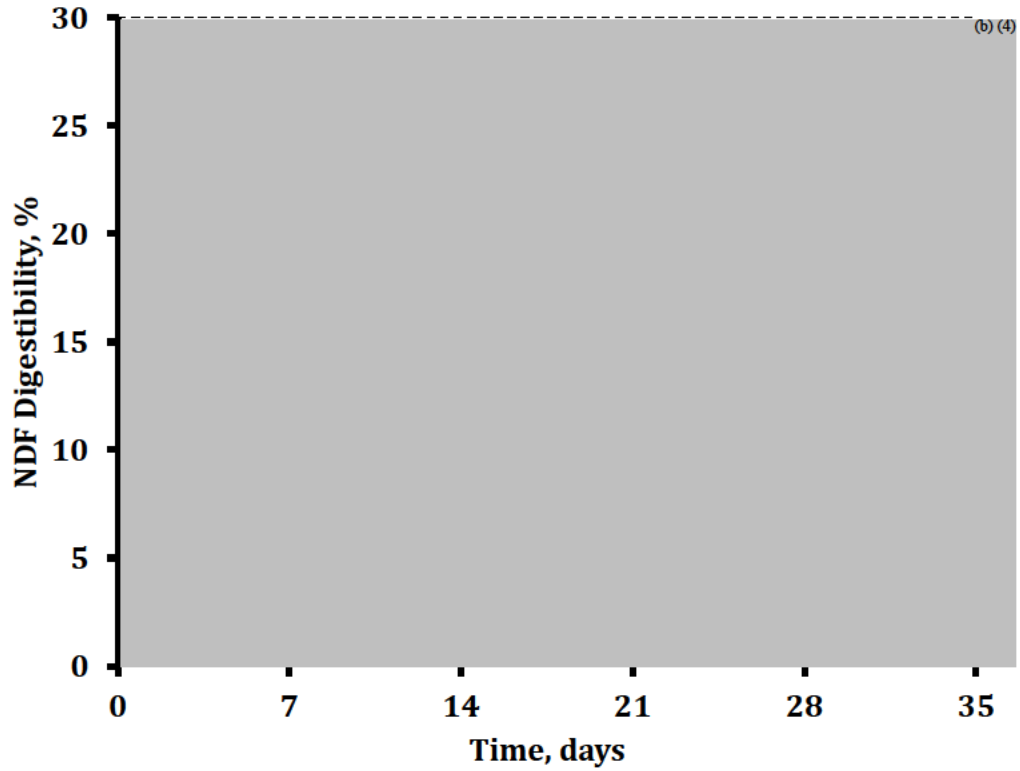
Figure III-9D: Apparent protein digestibility (%) covariate adjusted weekly least square means (± SEM) of cows assigned either to Control (circle) or Inoculated (trapezoid) by Intervention period study days. Effect of treatment (*P* = 0.0143) and treatment by time interaction (*P* = 0.8853). Treatment effect within week was established when a significant treatment by time interaction was observed (**P* < 0.10, ***P* < 0.05, ****P* < 0.01).
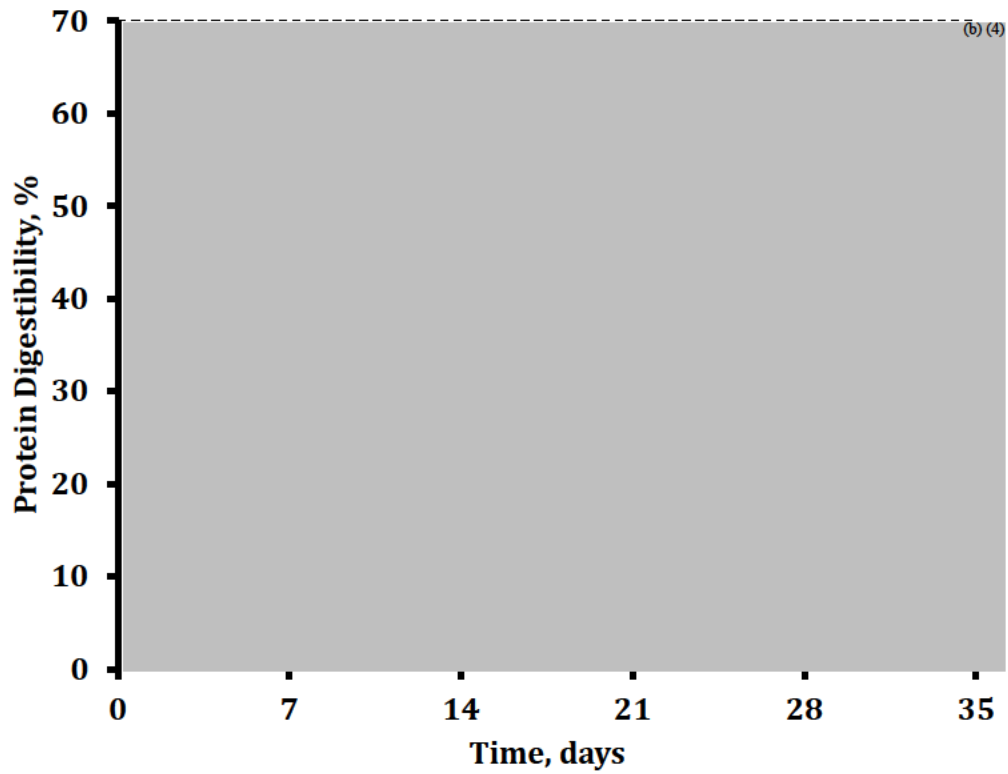
<u>Final In-Life Phase Report</u>

"Determine the Efficacy of Various Ascus Dairy Rumen Associated Microorganisms in Live Lactating Dairy Holstein Heifers via Direct Injection into the Rumen"

Study Number ⬛⬛ (b) (4)

Study Sponsor
Ascus Biosciences, Inc.
6450 Lusk Blvd
Suites E109 / 209
San Diego, CA 92121

In-Life Test Facility
⬛⬛⬛⬛⬛ (b) (4) ⬛⬛

Signature Page

"Determine the Efficacy of Various Ascus Dairy Rumen Associated Microorganisms in Live Lactating Dairy Holstein Heifers via Direct Injection into the Rumen"

(b) (4)

In-Life Phase Report

(b) (4)

_____ 29Aug 17
                           /Date

(b) (4)

Study Director

Table of Contents

Experiment Overview:

There were 3 treatment groups in the study. 8 experimental Holstein cows (average ~100 days in milk) received 2 microbes via injection into the rumen (Treatment Group 1: Dairy-20 & Dairy-21). 8 experimental Holstein cows (average ~100 days in milk) received 3 microbes via injection into the rumen (Treatment Group 2: Dairy- 10, Dairy-20 & Dairy-21). 8 experimental Holstein cows (average ~100 days in milk) received 3 basal suspension medias (no microbes) via injection into the rumen (Treatment Group 3: Control).

The cows were inoculated daily after the morning milking for 28 days. Fecal contents were sampled from each cow on study day 1 (prior to inoculation), and on study day 8, study day 16, study day 24, and study day 28. Samples had NDF and ADF determined. Feed samples were collected on Study Day 1 (prior to inoculation), and on Study Day 8, Study Day 16, Study Day 24, and Study Day 28. Samples had NDF and ADF determined. Rumen contents were sampled from each cow on Study Day 1 (prior to inoculation), and on Study Day 8, Study Day 16, Study Day 24, Study Day 28, Study Day 35 and Study Day 38. Twice daily milking, milk production measurements and clinical udder evaluations by quarter were performed every day from Study Day -7 to Study Day 38 for every individual animal, except for Cow 54027, which was not enrolled until Study Day 10, and for Cow 51005, which was removed from the study after Study Day 9. Both of these cows were removed from the statistical analyses. Cows were observed daily for overall clinical health from Study Day -7 to Study Day 38. Milk component measurements were taken on Study Days -7 to 38 in the AM and on Study Days 8 to 38 in the PM. Cows 54027 and 51005 were not included in the analysis.

Each individual cow was the experimental unit. The parameters statistically analyzed include the following:

- Fecal ADF, NDF, NDFom, and Dry Matter Percentage
- Feed ADF, NDF, NDFom, and Dry Matter Percentage
- Milk Production (Milk Production, Milk Fat Yield, Milk Protein Yield, Energy-Corrected Milk Yield, 3.5% Fat Corrected Milk Yield
- Milk Component Data: Milk Fat Percentage, Milk Protein Percentage, Milk Somatic Cell Count

Methods:

Research Candidate Evaluation

On Study Day -7, twenty-four lactation Holstein cows were evaluated for age, breed, parity, days in milk, duplicate ear tags with the same number, health, previous treatment history, disposition, udder with four good quarters.

Ration

The composition and calculated nutrient analysis for the ration fed during the study is in Attachment 1.

Ascus Representatives and Dairy Rumen Associated Microorganisms

Ascus Biosciences Laboratory provided the following:

- Sponsor Representatives
  Justin Wong
  Jordan Embree
- Ascus processed all microbes "in house", concentrations and re-suspensions were held in anaerobic vials on ice ready for administration.
- All negative control solutions were produced in the same manner.

Intra-Rumen Injection Administration

- An (b) (4) employee   (b) (4)) administered the daily intra-rumen injections to each cow.
- The intra-rumen injection site location was on the left side of the animal behind the last ribs in the paralumbar fossa. Prior to injection, each site was disinfected with isopropyl alcohol and allowed to dry.
- A 12-gauge 2-inch hypodermic needle was inserted through the abdominal wall and into the rumen. Afterwards, an 18-gauge, 6-inch spinal needle was inserted through the 12-gauge needle into the dorsal rumen.
- After needle insertion, intra-rumen location was confirmed by aspiration using a dose syringe.
- The Ascus representative gave syringes containing the appropriate microbes, or no microbes, to the dose administrator.

- After administration both needles inserted were removed and no further procedures were required.

Data and Samples Collected:

Measurements and Clinical Observations:

1. Twice daily milking, milk production measurements and clinical udder evaluations by quarter were performed every day for the full 38-day study period of the study for every individual animal.

   Milk measurements collected were as follows:

   Milk yield in pounds (Measured twice daily from Study Day -7 to 38).

   *Milk fat percentage. (Daily from Study Day -7 to 7 from the A.M. milking, and then twice daily to Study Day 38). Note: The Sponsor requested A.M. and P.M. milk sampling and measurements starting on Study Day 8.

   *Milk protein percentage.

   *Milk lactose percentage.

   *Milk solid percentage.

   *SCC (Somatic Cell Count).

      *Samples and measurements were daily from Study Day -7 to 7 from the A.M. milking, and then twice daily to Study Day 38. Note: The Sponsor requested A.M. and P.M. milk sampling and measurements starting on Study Day 8.

   Clinical udder evaluations were scored as follows:

   1=Normal Quarter/Normal Milk

   2=Normal Quarter/Questionable Milk

   3=Normal Quarter/Abnormal Milk

   4=Swollen Quarter/Abnormal Milk

   5= Swollen Quarter/Abnormal Milk/Systemic Abnormal Clinical Observations

2. Overall Clinical Health Observations:

Cows were observed daily for overall clinical health from Study Day -7 to Study Day 38.

3. Feed sampling:

Feed samples were collected on Study Day 1 (prior to inoculation), and on Study Day 8, Study Day 16, Study Day 24, and Study Day 28. Samples had NDF and ADF determined.

4. Fecal sampling:

Fecal contents were sampled from each cow on Study Day 1 (prior to inoculation), and on Study Day 8, Study Day 16, Study Day 24, and Study Day 28. Samples had NDF and ADF determined.

5. Rumen sampling:

Rumen contents were sampled from each cow on Study Day 1 (prior to inoculation), and on Study Day 8, Study Day 16, Study Day 24, Study Day 28, Study Day 35 and Study Day 38.

Rumen samples were collected via an orally inserted rumen tube.

Approximately 10 mL of rumen content was added to a conical containing Stop solution (prepared at Ascus, 15mL conicals were prefilled with 3 mL of stop solution)

Stop solution composition: 3 mL of ethanol containing 5% Trizol™

Hold sample conicals containing stop solution were stored at 4^0C until used.

At the time of sampling, each tube was sealed, then shake vigorously to disperse stop solution throughout rumen sample.

All tubes were stored at -20°C prior to shipment to Ascus Biosciences.

Statistical Analysis Methods:

All statistical comparisons of the treatment main effect and two-way interactions with the treatment main effect were performed at the 0.10 level of significance. Statistical analyses were performed using R statistical software version 3.4.0.

Fecal ADF and NDF

Fecal ADF (% DM), NDF (% DM), NDFom (% DM), and Dry Matter Percentage values from Study Days 1, 8, 16, 24, 28 were analyzed using the R package "nlme" and the lme function for linear mixed models, with treatment, study day, and treatment by study day interaction as fixed effects and Cow ID as a random effect (where appropriate).

fit <- lme (Response ~ Treatment_Group*Day, random = ~ 1 | ID, data=fecal_data)

Each model was compared to a fixed-effect only model using a Chi-squared test. Least square means were used to compare treatment groups using the unadjusted p-values and Satterthwaite degrees of freedom was used to test for significant differences. Tukey's method was used to adjust the p-values for multiple comparisons.

Milk Production

The daily total milk production data was transformed into four additional variables: Milk Fat Yield, Milk Protein Yield, Energy-Corrected Milk Yield, and 3.5% Fat Corrected Milk Yield. Milk Fat Yield was obtained using the following formula:

$$Milk\ Fat\ Yield = Milk\ Production\ (lbs) \times Milk\ Fat\ Percentage$$

Daily total milk production measurements were transformed into Milk Fat Yield using the average of the AM and PM Milk Fat Percentages for each cow on the corresponding Study Day. There were no PM Milk Fat Percentage measurements on Study Days 1 through 7, so the AM measurement was used for the calculation of Milk Fat Yield on these Study Days instead of the average.

Milk Protein Yield was obtained using the following formula:

$$Milk\ Protein\ Yield = Milk\ Production\ (lbs) \times Milk\ Protein\ Percentage$$

Daily total milk production measurements were transformed into Milk Protein Yield using the average of the AM and PM Milk Protein Percentages for each cow on the corresponding Study Day. There were no PM Milk Protein Percentage measurements on Study Days 1 through 7, so the AM measurement was used for the calculation of Milk Protein Yield on these Study Days instead of the average.

Energy-Corrected Milk Yield was obtained using the following formula:

$$ECM = 0.327 \times Milk\ Production\ (lbs) + 12.95 \times Milk\ Fat\ Yield + 7.2 \times Milk\ Protein\ Yield$$

Daily total milk production measurements were transformed into Energy-Corrected Milk Yield using the average of the AM and PM Milk Protein Percentages and the average of the AM and PM Milk Fat Percentages for each cow on the corresponding Study Day. There were no PM Milk

Protein Percentage or Milk Fat Percentage measurements on Study Days 1 through 7, so the AM measurements were used for the calculation of Energy-Corrected Milk Yield on these Study Days instead of the averages.

3.5% Fat Corrected Milk Yield was obtained using the following formula:

$$FCM = 0.432 \times Milk\ Production\ (lbs) + 16.23 \times Milk\ Fat\ Yield$$

Daily total milk production measurements were transformed into 3.5% Fat Corrected Milk Yield using the average of the AM and PM Milk Protein Percentages and the average of the AM and PM Milk Fat Percentages for each cow on the corresponding Study Day. There were no PM Milk Protein Percentage or Milk Fat Percentage measurements on Study Days 1 through 7, so the AM measurements were used for the calculation of 3.5% Fat Corrected Milk Yield on these Study Days instead of the averages.

Milk Production, Milk Fat Yield, Milk Protein Yield, Energy-Corrected Milk Yield, and 3.5% Fat Corrected Milk Yield measurements from Study Days 1 to 38 were analyzed using the R package "nlme" and the lme function for linear mixed models, with treatment, week (time period), and the treatment by week interaction term as fixed effects and Cow ID as a random effect (where appropriate).

    fit <- lme (Response ~ Trt_Group*Time_Period + (1 | Cow_ID), data=milk_data_prod, na.action = na.omit)

Each model was compared to a fixed-effect only model using a Chi-squared test. Least square means were used to compare treatment groups using the unadjusted p-values and Satterthwaite degrees of freedom was used to test for significant differences. Tukey's method was used to adjust the p-values for multiple comparisons.

Milk Component Data

Milk data (Milk Fat Percentage, Milk Protein Percentage, Milk Somatic Cell Count) measurements from Study Days 1 to 38 AM and 8 to 38 PM were analyzed using the R package "nlme" and the lme function for linear mixed models, with treatment, week (time period), and the treatment by week interaction terms as fixed effects and Cow ID as a random effect (where appropriate). AM and PM measurements were averaged per study day per cow for analysis. The data for Study Days 1 through 7 were only AM measurements.

    fit <- lme(Response ~ Trt_Group*Time_Period + (1 | Cow_ID), data=milk_data, na.action = na.omit)

Each model was compared to a fixed-effect only model using a Chi-squared test. Least square means were used to compare treatment groups using the unadjusted p-values and Satterthwaite degrees of freedom was used to test for significant differences. Tukey's method was used to adjust the p-values for multiple comparisons.

Feed Data

The feed data was a set of Dry Matter Percentage, ADF (% DM), NDF (% DM), and NDFom (% DM) values for samples taken on Study Days 1, 8, 16, 24, and 28. A summary table was produced for this data set.

Results:

Research Candidate Evaluation

On Study Day -7, twenty-four lactation Holstein cows that were 3-6 years-old, with a parity range of 2-4 lactations, 89-111 days in milk, with duplicate ear tags with the same number, good health, no previous medical treatment history within the previous 30 days, good disposition, and udder with four good quarters were selected for the study.

Ascus Representatives and Dairy Rumen Associated Microorganisms

Justin Wong and/or Jordan Embree were present on each day of dosing and presented the individual administering the intra-rumen injection with syringes containing the appropriate microbes or no microbes. Ascus processed all microbes "in house"; and each of the microbes was delivered at the dose of 1 X 10^9 CFUs/day.

Intra-Rumen Injection Administration

In general, the daily intra-rumen injections were administered uneventfully. Only small (<1 cm) injection site swellings were observed and were considered incidental.

Milk Production

Milk production (AM, PM and daily total) and milk component data (milk fat percentage, milk protein percentage, milk lactose percentage, milk solids percentage and milk somatic cell counts) measurements were taken on Study Days -7 to 38, but only the measurements from Study Days 1 to 38 were analyzed. The AM and PM measurements were pooled for analysis.

Variables are grouped by model outcome (where appropriate): non-significant TRT effect, significant TRT effect, significant TRT*Time Period (week) or TRT*Day effect. Statistically significant results for variables follow, when necessary. Only the appropriate differences are listed and significant differences are denoted with an asterisk (*).

Table 1 contains the Milk Production Data: Means by Study Day by Treatment Group. Table 2 contains the Milk Production (Prod) Data: Means by Time Period by Treatment Group. Table 3 contains Model Information for Milk Production Data. Table 4 contains Milk Production Data Differences for Treatment Effects. For Milk Production, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0185. Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 3, p=0.0754. Figure 1 shows the Graph of Weekly Least Square Means for Milk Production. Although the Treatment Group by Week interaction was significant, there were no significant individual Treatment Group LSMean differences within week for Milk Fat Yield. The adjustment for multiple comparisons created this disparity. Figure 2 shows the Graph of Weekly Least Square Means for Milk Fat Yield. For Milk Protein Yield, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0302. Figure 3 shows the Graph of Weekly Least Square Means for Milk Protein Yield. For Energy-corrected Milk Yield, Treatment Group 1 had significantly higher values than Treatment Group 3 during Week 2, p=0.0942. Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0303. Figure 4 shows the Graph of Weekly Least Square Means for Energy-Corrected Milk Yield. For 3.5% Fat-Corrected Milk Yield, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0405. Figure 5 shows the Graph of Weekly Least Square Means for 3.5% Fat Corrected Milk Yield.

Table 1 Milk Production (Prod:lbs) Data: Means by Study Day by Treatment Group

Study Day	Average of AM Milk Prod			Average of PM Milk Prod			Average of Daily Total Milk Prod		
	T1	T2	T3	T1	T2	T3	T1	T2	T3
-7	56.875	62.250	57.750	33.500	30.875	31.750	90.375	93.125	89.500
-6	51.750	46.750	50.500	45.125	47.000	45.625	96.875	93.750	96.125
-5	57.000	58.250	60.625	45.500	47.875	43.375	102.500	106.125	104.000
-4	55.625	56.750	55.375	46.000	47.500	42.750	101.625	104.250	98.125
-3	54.500	55.000	55.375	47.625	46.250	45.500	102.125	101.250	100.875
-2	56.625	58.000	57.750	46.750	45.750	40.500	103.375	103.750	98.250
-1	63.875	61.375	56.000	41.375	47.875	47.625	105.250	109.250	103.625
1	55.250	56.750	54.375	41.500	48.750	47.500	96.750	105.500	101.875
2	45.000	53.375	53.000	44.250	46.500	43.250	89.250	99.875	96.250
3	55.500	60.125	50.375	42.000	47.500	43.125	97.500	107.625	93.500
4	54.375	60.500	56.750	42.500	49.750	45.875	96.875	110.250	102.625
5	48.125	60.375	56.000	45.000	52.250	46.500	93.125	112.625	102.500
6	53.750	57.000	54.875	45.750	53.250	43.500	99.500	110.250	98.375
7	51.750	60.000	58.125	43.750	50.500	45.750	95.500	110.500	103.875
8	52.750	59.750	50.000	47.625	50.375	41.750	100.375	110.125	91.750
9	50.000	57.750	51.125	45.625	50.250	41.500	95.625	108.000	92.625
10	56.875	58.000	45.750	52.500	52.500	41.250	109.375	110.500	87.000
11	54.250	56.250	48.125	47.625	52.500	43.500	101.875	108.750	91.625
12	53.625	50.500	46.250	45.875	47.875	39.875	99.500	98.375	86.125
13	54.875	56.125	45.500	43.500	49.000	43.375	98.375	105.125	88.875
14	53.000	56.500	44.875	46.375	50.000	43.250	99.375	106.500	88.125
15	56.750	55.500	50.500	48.000	53.625	45.500	104.750	109.125	96.000
16	55.750	58.500	48.125	48.125	51.375	44.625	103.875	109.875	92.750
17	54.750	55.000	46.500	49.875	46.750	43.000	104.625	101.750	89.500
18	56.500	58.750	50.375	46.000	47.250	42.250	102.500	106.000	92.625
19	54.500	53.375	47.750	47.250	51.500	46.000	101.750	104.875	93.750
20	57.875	55.500	47.750	48.375	51.000	43.875	106.250	106.500	91.625
21	53.500	57.125	51.875	46.125	48.500	41.750	99.625	105.625	93.625
22	55.125	56.375	49.250	42.125	47.000	44.375	97.250	103.375	93.625
23	56.625	56.375	52.625	46.625	47.500	41.750	103.250	103.875	94.375
24	52.625	54.125	52.250	45.750	49.500	45.875	98.375	103.625	98.125
25	48.625	55.250	46.875	46.750	50.625	50.000	95.375	105.875	96.875
26	50.375	54.500	50.000	44.250	47.250	45.250	94.625	101.750	95.250
27	53.625	56.375	54.375	43.125	43.375	45.375	96.750	99.750	99.750
28	50.125	52.000	51.875	43.250	45.000	41.875	93.375	97.000	93.750
29	47.125	47.500	49.125	42.500	41.625	42.000	89.625	89.125	91.125
30	49.500	49.625	49.625	43.750	46.500	44.125	93.250	96.125	93.750
31	51.000	48.250	48.125	43.375	40.625	40.000	94.375	88.875	88.125
32	51.625	50.250	49.000	42.750	45.250	41.500	94.375	95.500	90.500
33	56.125	49.500	51.250	46.125	47.500	45.125	102.250	97.000	96.375
34	52.750	51.375	51.625	47.875	45.250	43.625	100.625	96.625	95.250
35	51.125	53.000	52.000	43.500	44.250	45.750	94.625	97.250	97.750
36	44.125	50.625	54.250	41.750	42.000	46.500	85.875	92.625	100.750
37	49.625	54.625	52.750	40.500	45.000	43.875	90.125	99.625	96.625
38	51.125	49.250	52.875	38.375	42.750	42.375	89.500	92.000	95.250

Table 2 Milk Production (Prod:lbs) Data: Means by Time Period by Treatment Group

Time Period	Average of AM Milk Prod			Average of PM Milk Prod			Average of Daily Total Milk Prod		
	T1	T2	T3	T1	T2	T3	T1	T2	T3
Baseline	56.607	56.911	56.196	43.696	44.732	42.446	100.304	101.643	98.643
Treatment	53.424	56.491	50.545	45.696	49.330	43.982	99.121	105.821	94.527
Post-Treatment	50.413	50.400	51.063	43.050	44.075	43.488	93.463	94.475	94.550

Table 3 Model Information for Milk Production Data, Study Day Model

| Variable	Model Type	P-values		Decision
		Treatment_Group	Treatment_Group*Week	
Milk Production	Mixed	0.3233	<0.0001	(3)
Milk Fat Yield	Mixed	0.637	0.022	(3)
Milk Protein Yield	Mixed	0.5017	<0.0001	(3)
Energy-Corrected Milk Yield	Mixed	0.4284	<0.0001	(3)
3.5% Fat-Corrected Milk Yield	Mixed	0.4348	<0.0001	(3)

Decisions:

(1) There were no significant terms involving Treatment Group. No further evaluation is needed.

(2) The Treatment Group main effect is significant at $\alpha=0.10$, and the 2-way interaction is not significant. Compare treatment means from the main effect of Treatment Group.

(3) The Treatment Group by Week interaction is significant at $\alpha=0.10$. Compare treatment means within each week.

## Table 4 Decision (3) Milk Production Data Differences and Standard Deviations for Treatment Effects

Variable	Week	Compare	Difference	Standard Error	P-value
Milk Production	2	Treatment Group 1 vs. Treatment Group 3	6.4707	2.9723	0.0998
Milk Production	2	Treatment Group 2 vs. Treatment Group 3	8.6607	2.8715	0.0179
Milk Production	3	Treatment Group 2 vs. Treatment Group 3	6.7054	2.8715	0.0737
Milk Fat Yield	1	Treatment Group 1 vs Treatment Group 2	26.5311	11.9021	0.0905
Milk Protein Yield	1	Treatment Group 1 vs Treatment Group 2	21.5625	7.5398	0.0251
Milk Protein Yield	1	Treatment Group 2 vs Treatment Group 3	-16.1260	7.2853	0.0932
Energy-Corrected Milk Yield	2	Treatment Group 2 vs Treatment Group 3	520.3163	214.2768	0.0614
3.5% Fat-Corrected Milk Yield	1	Treatment Group 1 vs Treatment Group 2	433.2352	193.6081	0.0890

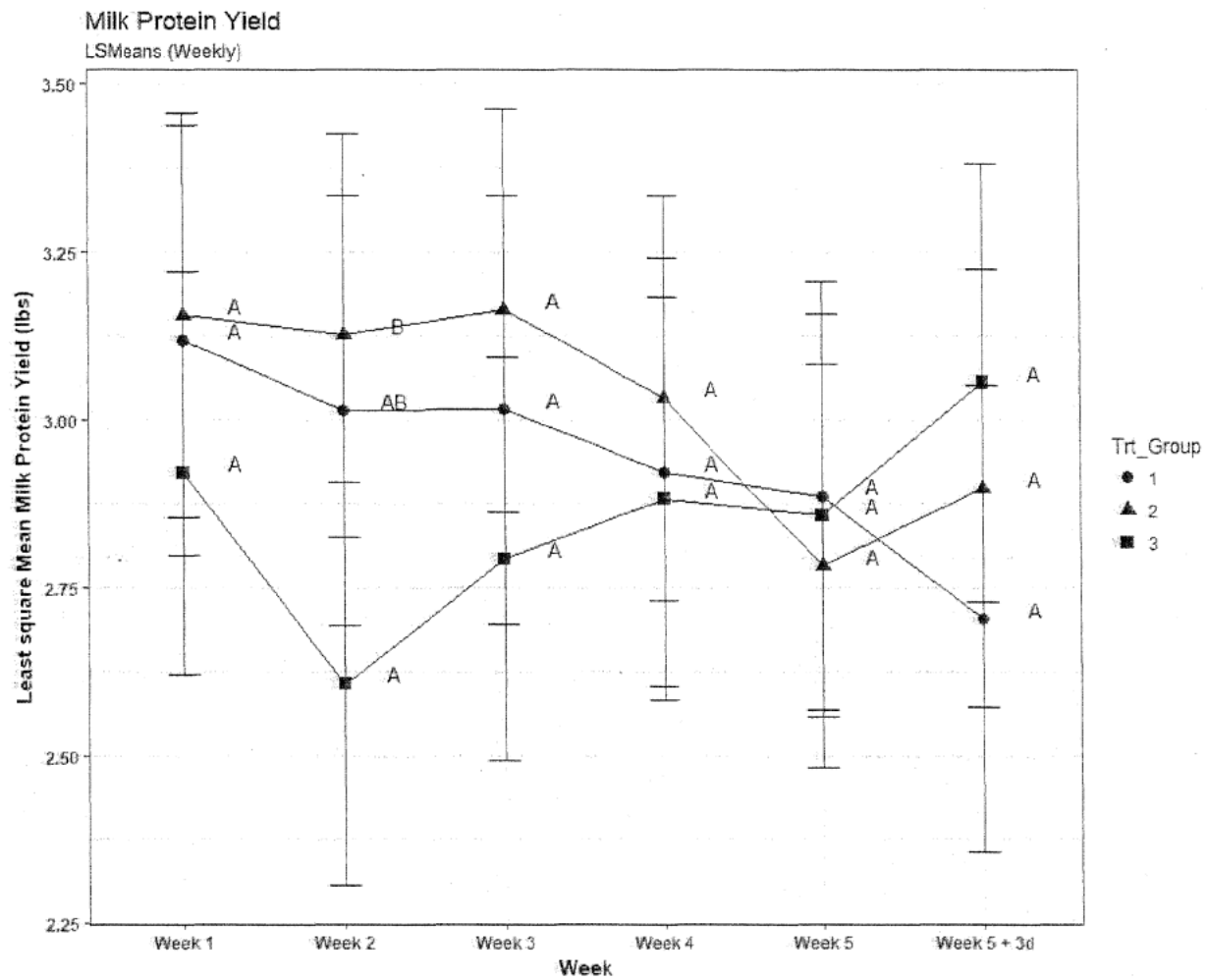# Figure 1: Graph of Weekly Least Square Means for Milk Production
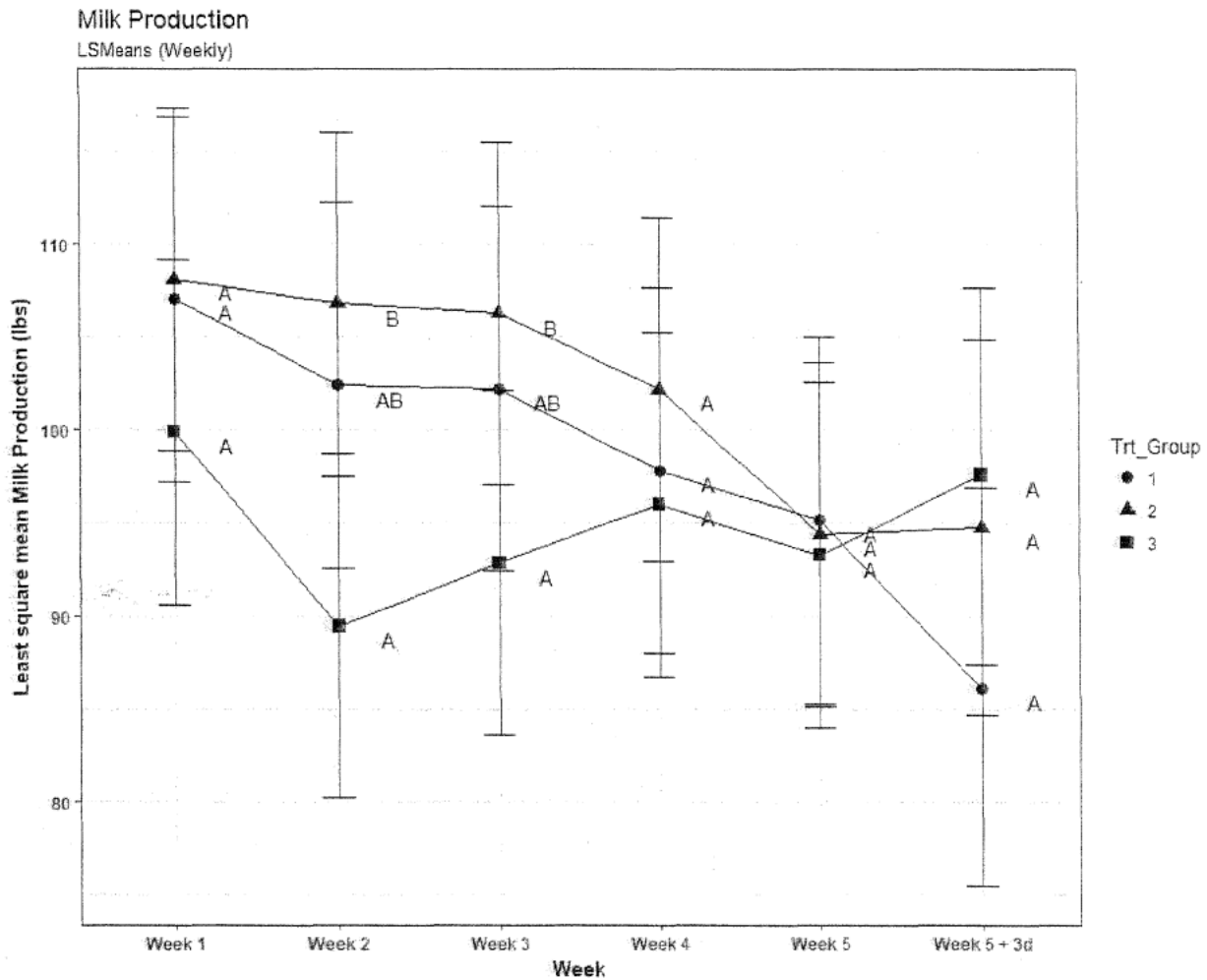
Milk Production (daily total, lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Figure 2: Graph of Weekly Least Square Means for Milk Fat Yield
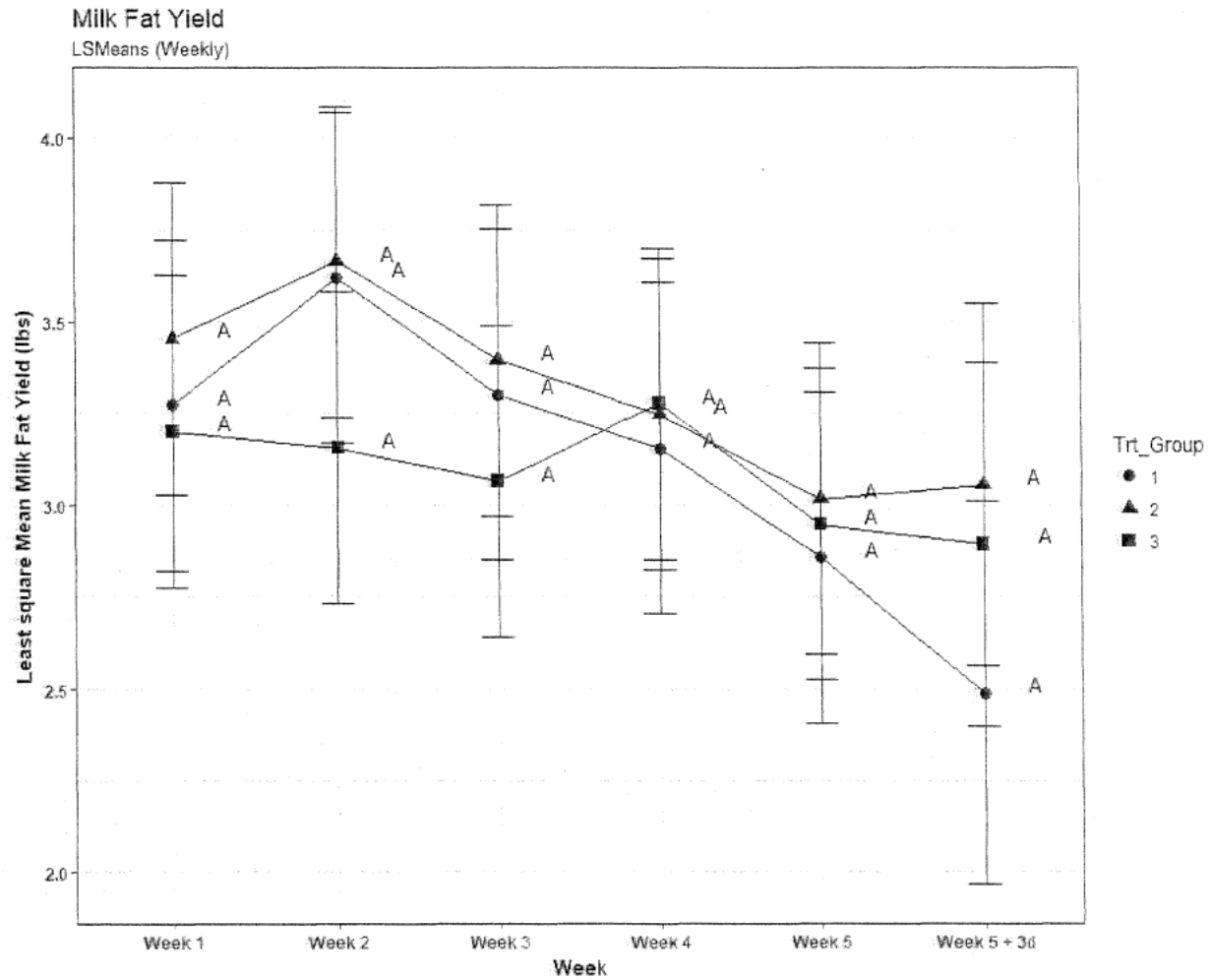
Milk Fat Yield

LSMeans (Weekly)

Milk Fat Yield (lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

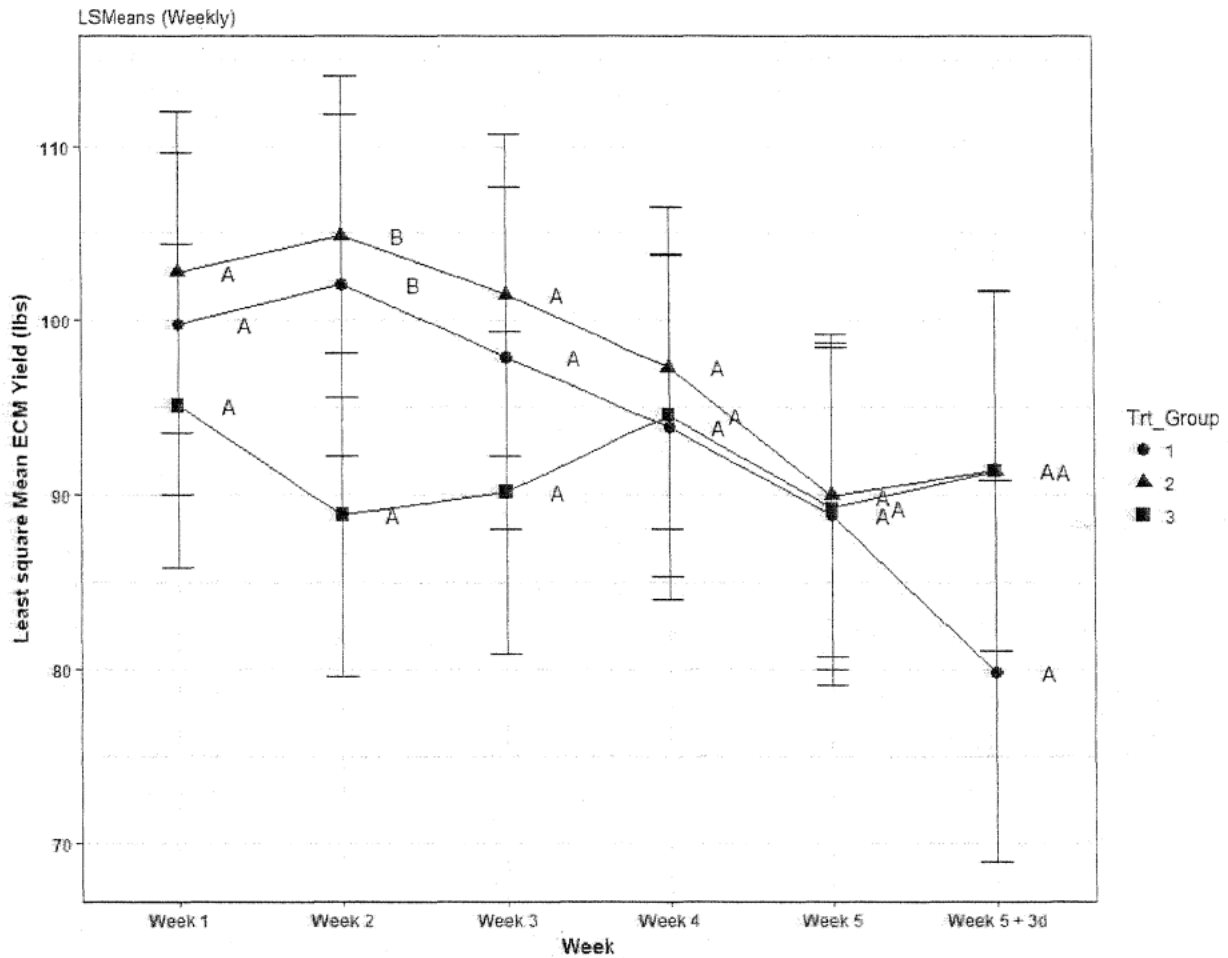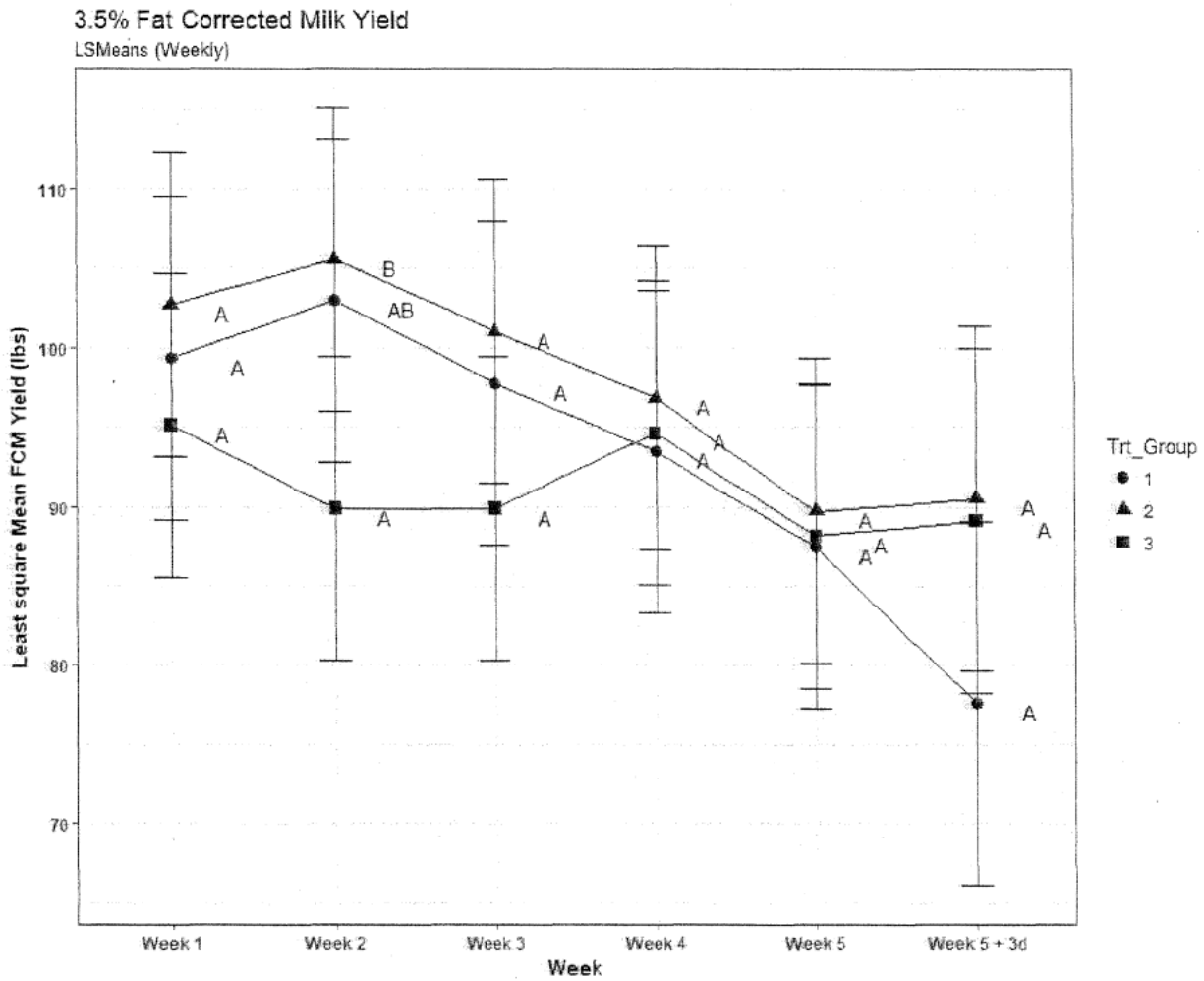## Figure 3: Graph of Weekly Least Square Means for Milk Protein Yield

Milk Protein Yield (lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Figure 4: Graph of Weekly Least Square Means for Energy-Corrected Milk Yield

Energy-Corrected Milk Yield (lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Figure 5: Graph of Weekly Least Square Means for 3.5% Fat Corrected Milk Yield

3.5% Fat Corrected Milk Yield

LSMeans (Weekly)

3.5% Fat Corrected Milk Yield (lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Milk Component Data

Milk data (Milk Fat Percentage, Milk Protein Percentage, Milk Lactose Percentage, Milk Solids Percentage, Milk Somatic Cell Count) were measured on Study Days -7 to 38 for AM measurements, and on Study Days 8 to 38 for PM measurements. The milk data from Study Days 1 to 38 were analyzed. AM and PM measurements were averaged per study day per cow for analysis. The data for Study Days 1 through 7 were only AM measurements. Table 5 contains Model Information for Milk Component Data. Table 6 contains Milk Component Data for treatment effects. There were no significant individual Treatment Group LSMean differences within week for Milk Fat %. Figure 6 show the Graph of Weekly Least Square Means for Milk Fat Percentage. For Milk Protein %, Treatment Group 1 had significantly higher values than Treatment Group 2 during Week 5 + 2d, p=0.0001. Treatment Group 1 had significantly higher values than Treatment Group 3 during Week 5 + 2d, p=0.0009. Figure 7 shows the Graph of Weekly Least Square Means for Milk Protein Percentage. For Milk SCC, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 1, p=0.0273. Figure 8 shows the Graph of Weekly Least Square Means for Milk Somatic Cell Count.

Table 5: Model Information for Milk Component Data

| Variable	Model Type	P-values		Decision
		Treatment Group	Treatment Group*Week	
Milk Fat %	Mixed	0.8392	0.1733	(1)
Milk Protein %	Mixed	0.7404	<0.0001	(3)
Milk SCC	Mixed	0.1310	0.0218	(3)

Decisions:
(1) There were no significant terms involving Treatment_Group. No further evaluation is needed.
(2) The Treatment_Group main effect is significant at α=0.10, and the 2-way interaction is not significant. Compare treatment means from the main effect of Treatment_Group.
(3) The Treatment_Group by Week interaction is significant at α=0.10. Compare treatment means within each week.

Table 6: Milk Component Data for Treatment Effects

Variable	Week	Compare	Difference	Standard Error	P-value
Milk Protein %	5 + 2d	Treatment Group 1 vs. Treatment Group 2	0.5731	0.1051	0.0001
Milk Protein %	5 + 2d	Treatment Group 1 vs. Treatment Group 3	0.4569	0.1051	0.0009
Milk SCC	1	Treatment Group 2 vs Treatment Group 3	1.494	0.5299	0.0273

Figure 6: Graph of Weekly Least Square Means for Milk Fat Percentage
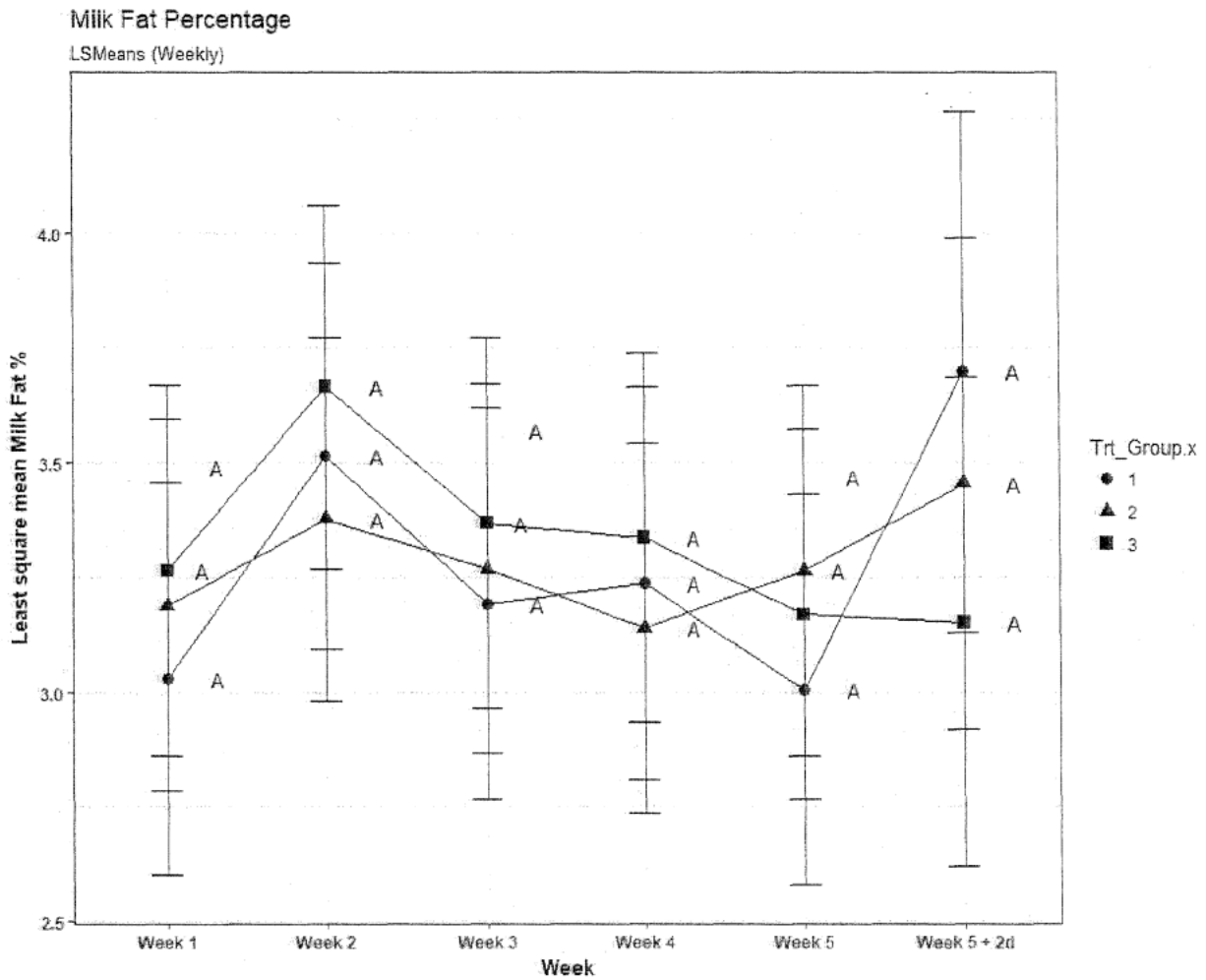
Milk Fat Percentage for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Figure 7: Graph of Weekly Least Square Means for Milk Protein Percentage
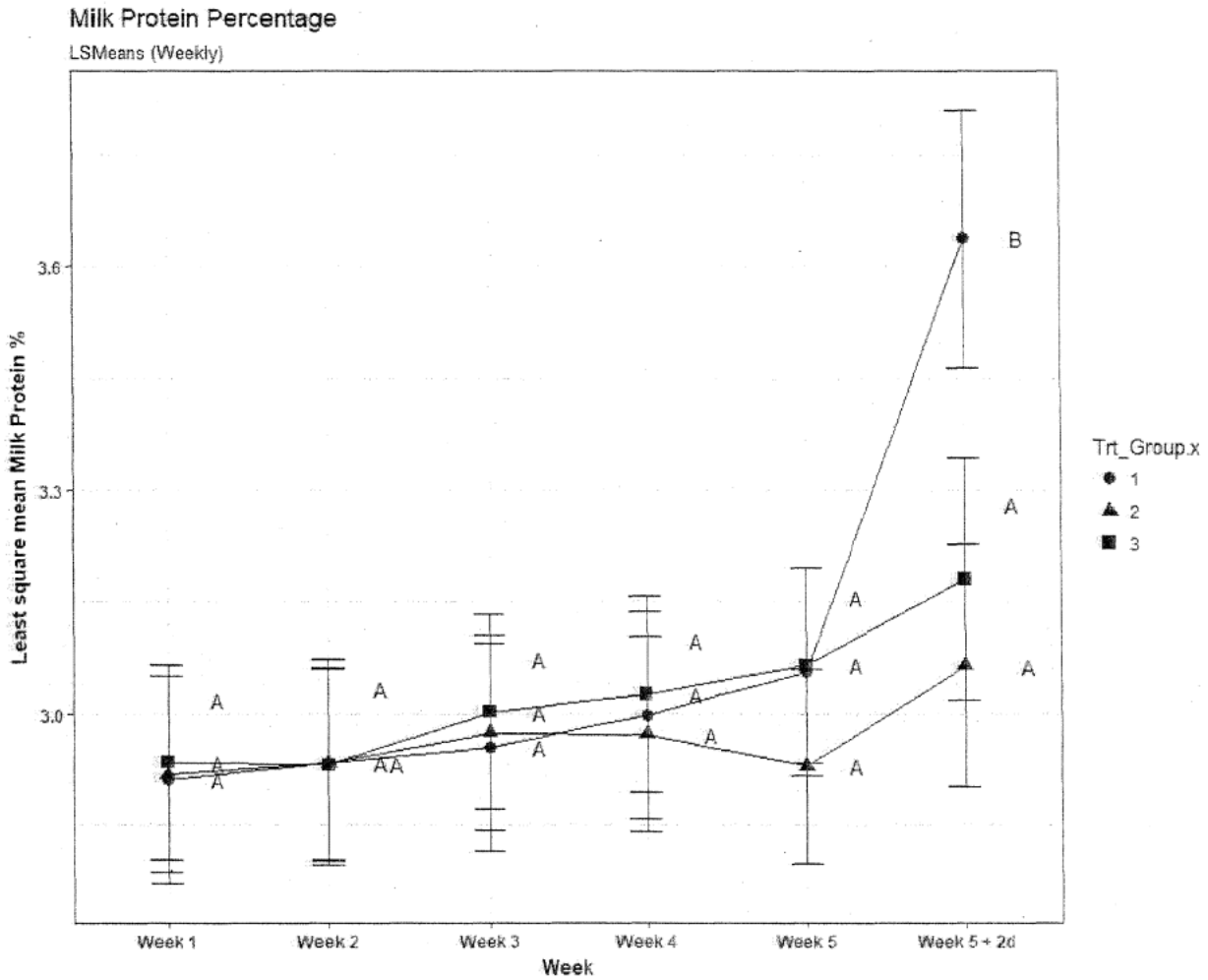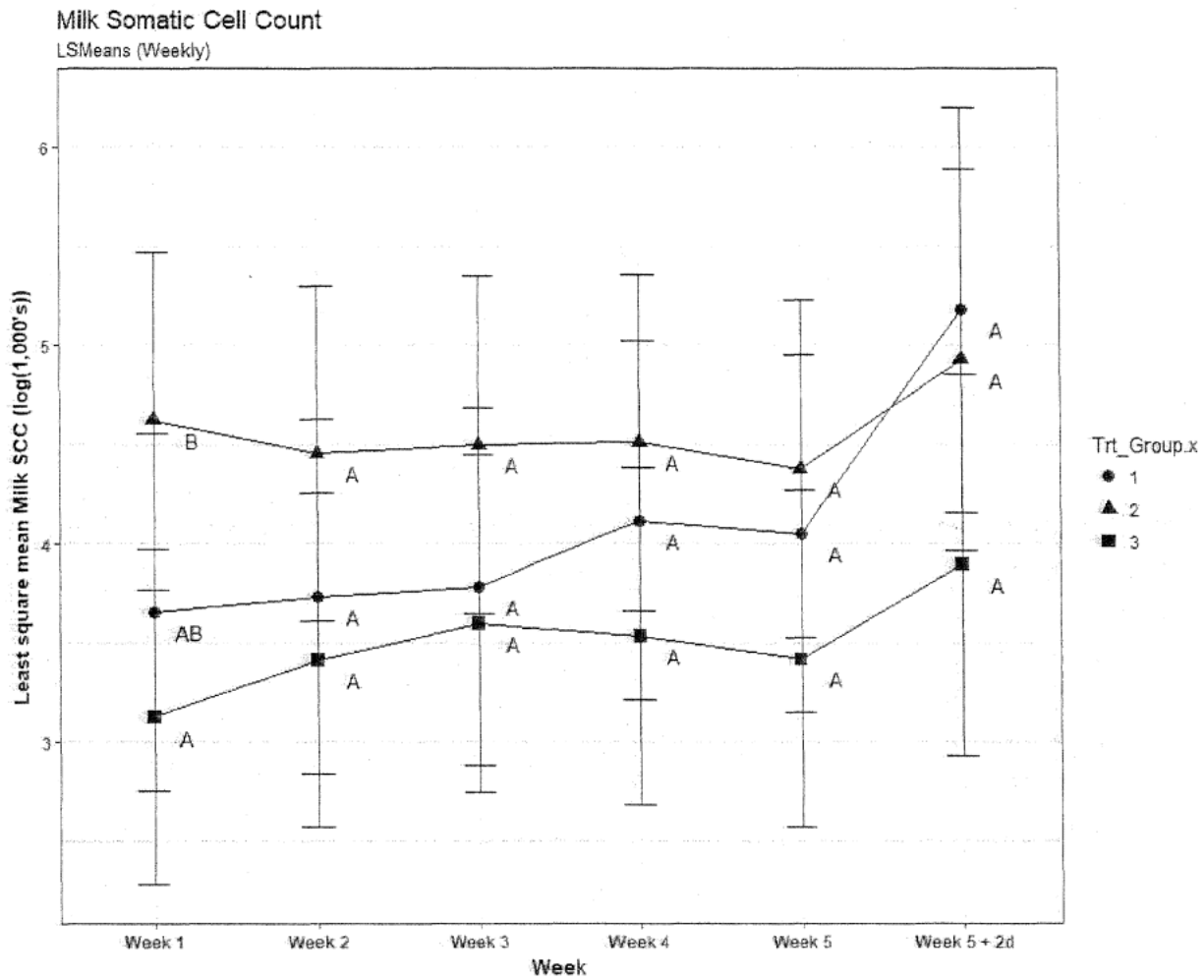
Milk Protein Percentage

Milk Protein Percentage for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Figure 8: Graph of Weekly Least Square Means for Milk Somatic Cell Count

Milk Somatic Cell Count (log(1,000's)) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Feed Data

For feed, Dry Matter Percentage, ADF (% DM), NDF (% DM), and NDFom (% DM) values for samples were measured on Study Days 1, 8, 16, 24, and 28. Table 7 shows the Summary of Feed Data.

Table 7: Summary of Feed Data

	Dry Matter Percentage	ADF (% DM)	NDF (% DM)	NDFom (% DM)
Minimum	0.4654	0.1901	0.2764	0.2587
1st Quartile	0.4755	0.1978	0.2835	0.2636
Median	0.4847	0.1985	0.2879	0.2649
Mean	0.4813	0.1982	0.2861	0.2648
3rd Quartile	0.4855	0.1998	0.2892	0.2667
Maximum	0.4952	0.2046	0.2937	0.2702
Standard Deviation	0.01128	0.005229	0.006547	0.004226
Coefficient of Variation (%)	2.344	2.639	2.288	1.596

Clinical Udder Evaluations

Abnormal clinical udder findings were considered minimal, incidental and not treatment group-related during the study.

Cow #51005, Treatment Group 1, had one episode of mastitis from Study Day 1 to 9 (multiple quarters scored 4-2), and did not respond well to Spectromast LC® (Zoetis) intra-mammary antimicrobial treatment. This cow was replaced with cow 54027 on Study Day 10.

Cow #49155, Treatment Group 1, had one episode of mastitis on Study Days 35 to 38 (multiple quarters scored 4-2) and was treated with Spectromast LC® (Zoetis) intra-mammary antimicrobial treatment. This cow did not respond well to intra-mammary treatment, but completed the study.

Cow #47520, Treatment Group 2, had two episodes of mastitis, the first on Study Days 2-4 (one quarter scored 4-2) and was treated with Spectromast LC® (Zoetis) intra-mammary antimicrobial treatment. This cow responded well to intra-mammary treatment and returned to normal. The second episode of mastitis was on Study Days 29 to 38 (one quarter scored 3-2), and was not treated and completed the study.

Cow #49654, Treatment Group 2, had two episodes of mastitis, the first on Study Days 11-14 (one quarter scored 3-2) and was not treated and this cow returned to normal. The second episode of mastitis was on Study Days 22 to 24 (the same one quarter scored 3-2), and was not treated, returned to normal and completed the study.

Cow #53110, Treatment Group 3, had one episode of mastitis on Study Days 2 to 7 (one quarter scored 4-2) and was treated with Spectromast LC® (Zoetis) intra-mammary antimicrobial treatment. This cow responded well to intra-mammary treatment, and completed the study.

Overall Clinical Health Observations

Abnormal clinical health observations, as determined by observing the cows in their pen) were considered minimal, incidental and not treatment group-related during the study.

All animals were clinically normal from Study Day -7 to 35 (except for mastitis cases stated above, which were determined at the time of milking.

On Study Days 36 to 38 two cows, #51562, Treatment Group 2 and #49155, Treatment Group 1, were both observed depressed. Cow #49155 had an episode of mastitis ongoing and was being treated with Spectromast LC® (Zoetis) intra-mammary antimicrobial treatment (described above). Both animals completed the study. Cow #51562 did not have mastitis and depression was the only abnormal clinical observation and considered most likely due to focal local injection site inflammation due to the multiple intra-rumen injections.

Fecal ADF and NDF

Fecal ADF (% DM), NDF(% DM), NDFom (% DM), and Dry Matter Percentage were measured from Study Days 1, 8, 16, 24 and 28. Table 8 contains the model information for Fecal Data. Table 9 contains Fecal Data Dry Matter % Differences for Treatment Effects. For Fecal Data Dry Matter Percentage, Treatment Group 1 had significantly higher values than Treatment Group 3, p=0.0229. Table 10 contains the Fecal Data NDF (%DM) Differences for Treatment Effects. For NDF (% DM), Treatment Group 1 had significantly higher values than Treatment Group 2 on Day 1, p=0.0146. Treatment Group 2 had significantly lower values than Treatment Group 3 on Day 1, p=0.0631.

Table 8: Model Information for Fecal Data

Variable	Model Type	P-values		Decision
		Treatment Group	Treatment Group*Study Day	
ADF (% DM)	Fixed Effects Only	0.2433	0.1497	(1)
NDF (% DM)	Fixed Effects Only	0.2833	0.05478	(3)
NDFom (% DM)	Fixed Effects Only	0.2386	0.1796	(1)
Dry Matter %	Fixed Effects Only	0.03432	0.1777	(2)

Decisions:

(1) There were no significant terms involving Treatment Group. No further evaluation is needed.

(2) The Treatment Group main effect is significant at $\alpha=0.10$, and the 2-way interaction is not significant. Compare treatment means from the main effect of Treatment Group.

(3) The Treatment Group by Study Day interaction is significant at $\alpha=0.10$. Compare treatment means within each day.

Table 9 Fecal Data Dry Matter % Differences for Treatment Effects

Variable	Compare	Difference	Standard Error	P-value	Significance
Dry Matter %	Treatment Group 1 vs. Treatment Group 2	0.005060	0.005165	0.5914	
Dry Matter %	Treatment Group 1 vs. Treatment Group 3	0.01416	0.005272	0.0229	*
Dry Matter %	Treatment Group 2 vs. Treatment Group 3	0.009096	0.005201	0.1923	

Table 10 Fecal Data NDF (% DM) Differences for Treatment Effects

Variable	Study Day	Compare	Difference	Standard Error	P-value	Significance
NDF (% DM)	1	Treatment Group 1 vs. Treatment Group 2	0.1081	0.03793	0.0146	*
NDF (% DM)	1	Treatment Group 2 vs. Treatment Group 3	-0.08360	0.03665	0.0631	*

Rumen Samples

Rumen samples were submitted to the Sponsor for evaluation and the results are not reported in this report.

Conclusions:

In the opinion of the Investigator, abnormal clinical udder findings and abnormal clinical health observations were considered minimal, incidental and not treatment group-related during the study.

Statistically significant differences between treatment groups were determined to be as follows:

For Fecal Data Dry Matter Percentage, Treatment Group 1 had significantly higher values than Treatment Group 3, p=0.0229.

For Fecal Data NDF (% DM), Treatment Group 1 had significantly higher values than Treatment Group 2 on Day 1, p=0.0146. Treatment Group 2 had significantly lower values than Treatment Group 3 on Day 1, p=0.0631.

For Milk Protein %, Treatment Group 1 had significantly higher values than Treatment Group 2 during Week 5 + 2d, p=0.0001. Treatment Group 1 had significantly higher values than Treatment Group 3 during Week 5 + 2d, p=0.0009.

For Milk SCC, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 1, p=0.0273.

For Milk Production, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0185. Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 3, p=0.0754.

For Milk Fat Yield, Treatment Group 1 had significantly higher values than Treatment Group 2 during Week 1, p=0.0905.

For Milk Protein Yield, Treatment Group 1 had significantly higher values than Treatment Group 2 during Week 1, p=0.0251. Treatment Group 2 had significantly lower values than Treatment Group 3 during Week 1, p=0.0932.

For Energy-corrected Milk Yield, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0614.

For 3.5% Fat-Corrected Milk Yield, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0405.

Attachment 1 Ration Composition and Calculated Nutrient Analysis

(b) (4)

Ration Outputs (Fresh Cows)

AMTS.Cattle.Professional

Farm: LoneOak
Cattle: Fresh Cows

FBW: 1550 lbs
BCS (1-5): 3.00
ADG: 0.000 lbs/day

DIM: 30
Milk: 84.9 lbs/day
Milk Fat: 3.70%
Milk Prt: 3.10%

Inputted DMI: 52.41 lbs
Predicted DMI: 40.76 lbs

Ration Fed				
Ingredient	$/hd	%DM	DM lbs/day	AF lbs/day
Alfalfa Hay 20 CP 37 NDF 17 LNDF	1.53	90.0	11.00	12.22
Corn Silage	1.10	35.1	12.46	35.50
Corn	0.41	85.0	3.00	3.53
EnerGII Regular	0.31	98.0	0.60	0.61
High Moisture Corn 30%	0.48	70.3	3.50	4.98
Soy Plus	0.41	89.1	1.37	1.53
Molasses Cane	0.08	71.0	0.64	0.90
Almond Hulls-Alpha Dairy Consulting	0.10	89.0	1.28	1.44
Canola	0.91	90.0	4.27	4.74
Cottonseed	0.63	91.0	3.20	3.52
LO MC Min 070912	0.23	98.5	1.28	1.30
Wheat Straw 5 CP 79 NDF 16 LNDF	0.10	92.0	1.71	1.86
Soyhull Pellets	0.46	90.0	4.27	4.74
DDG	0.67	89.0	3.84	4.32
Totals	7.43	64.6	52.41	81.19

Cost/ton As-Fed: $183.04

Output	Min	Value	Max	Status
Cost/hd	2.75	7.43	100.00	OK
DM (%)	20.00	64.56	80.00	OK
Dry Matter Intake (lbs/day)	24.60	52.41	24.70	HIGH
Forage NDF (%NDF)	0.00	57.57	100.00	OK
Forage (%DM)	0.00	48.02	100.00	OK
ADF (%DM)	0.00	25.71	100.00	OK
NDF (%DM)	0.00	37.25	100.00	OK
peNDF (%DM)	22.00	25.28	35.00	OK
ME Allowable Milk (lbs/day)	84.04	66.47	85.74	LOW
MP Allowable Milk (lbs/day)	84.04	78.45	85.74	LOW
ME (%Rqd)	99.00	85.49	101.00	LOW
MP (%Rqd)	99.00	95.12	101.00	LOW
CP (%)	-	16.32	-	-
SP (%CP)	-	31.81	-	-
RDP (%DM)	-	8.53	-	-
NFC (%DM)	0.00	31.75	40.00	OK
Sugar (%DM)	0.00	6.10	12.00	OK
Starch (%DM)	0.00	17.11	30.00	OK
Soluble Fiber (%DM)	0.00	6.79	10.00	OK
EE (%DM)	0.00	5.76	6.50	OK
LCFA (%DM)	0.00	4.74	6.50	OK
Total Unsaturate (%DM)	0.00	3.30	3.00	HIGH
NEl (Mcal/lb)	-	0.67	-	-
DCAD1 (meq/kg)	-200.00	305.89	500.00	OK
MP Supply (g)	500.00	2633.39	3000.00	OK
CHO-C (g)	0.00	3102.24	2200.00	HIGH
Ferm. CHO (%DM)	10.00	36.19	70.00	OK
Fermentable CHO (%CHO)	0.00	53.14	70.00	OK
IOFC	0.00	-7.43	100.00	LOW
Ca (g)	0.00	212.27	200.00	HIGH
Ca (%DM)	0.00	0.89	2.00	OK
Mg (%DM)	0.00	0.34	2.00	OK
P (%DM)	0.00	0.40	2.00	OK
K (%DM)	0.00	1.49	2.00	OK
S (%DM)	0.00	0.26	2.00	OK
Na (%DM)	0.00	0.44	2.00	OK
Cl (%DM)	0.00	0.39	2.00	OK
Vit-A (KIU)	0.00	64.56	110.00	OK
Vit-D (KIU)	0.00	11.39	50.00	OK
Vit-E (IU)	0.00	172.16	3000.00	OK
LYS (%MP)	0.00	6.29	7.60	OK
MET (%MP)	0.00	1.97	2.40	OK
LYS:MET	2.80	3.19	4.00	OK
ME (Mcal/lb)	-	1.04	-	-
NEg (Mcal/lb)	-	0.38	-	-
NEm (Mcal/lb)	-	0.65	-	-
Monensin (mg/day)	0.00	225.25	480.00	OK
IOpurFC	-	-4.52	-	-
Purchased Cost/hd	-	4.52	-	-
Total Manure N (g)	0.00	0.00	100.00	OK
Total Manure P (g)	0.00	0.00	100.00	OK

Attachment 2 Statistical Report

Attachment 2 Statistical Report

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Determine the Efficacy of Various Ascus Dairy Rumen Associated Microorganisms
In Live Lactating Dairy Holstein Heifers via Direct Injection into the Rumen

Statistical Report

"Determine the Efficacy of Various Ascus Dairy Rumen Associated Microorganisms in Live Lactating Dairy Holstein Heifers via Direct Injection into the Rumen"

Study Number HMS 020617

Study Sponsor:

Ascus Biosciences, Inc.

6450 Lusk Blvd

Suites E109 / 209

San Diego, CA 92121

In-Life Test Facility:

(b) (4)

Prepared by:

(b) (4)

_____ Date: 04 August 17

(b) (4)

Determine the Efficacy of Various Ascus Dairy Rumen Associated Microorganisms
In Live Lactating Dairy Holstein Heifers via Direct Injection into the Rumen

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Determine the Efficacy of Various Ascus Dairy Rumen Associated Microorganisms
In Live Lactating Dairy Holstein Heifers via Direct Injection into the Rumen

1.0   INTRODUCTION

There were 3 treatment groups in the study. 8 experimental Holstein cows (average ~100 days in milk) received 2 microbes via injection into the rumen (Treatment Group 1). 8 experimental Holstein cows (average ~100 days in milk) received 3 microbes via injection into the rumen (Treatment Group 2). 8 experimental Holstein cows (average ~100 days in milk) received 3 basal suspension medias (no microbes) via injection into the rumen (Treatment Group 3).

The cows were inoculated daily after the morning milking for 28 days. Fecal contents were sampled from each cow on study day 1 (prior to inoculation), and on study day 8, study day 16, study day 24, and study day 28. Samples had NDF and ADF determined. Feed samples were collected on Study Day 1 (prior to inoculation), and on Study Day 8, Study Day 16, Study Day 24, and Study Day 28. Samples had NDF and ADF determined. Rumen contents were sampled from each cow on Study Day 1 (prior to inoculation), and on Study Day 8, Study Day 16, Study Day 24, Study Day 28, Study Day 35 and Study Day 38. Twice daily milking, milk production measurements and clinical udder evaluations by quarter were performed every day from Study Day -7 to Study Day 38 for every individual animal, except for Cow 54027, which was not enrolled until Study Day 10, and for Cow 51005, which was removed from the study after Study Day 9. Cows were observed daily for overall clinical health from Study Day -7 to Study Day 38. Milk component measurements were taken on Study Days -7 to 38 in the AM and on Study Days 8 to 38 in the PM. Cows 54027 and 51005 were not included in the analysis.

Each individual cow was the experimental unit. The parameters statistically analyzed include the following:

- Fecal ADF, NDF, NDFom, and Dry Matter Percentage
- Feed ADF, NDF, NDFom, and Dry Matter Percentage
- Milk Production (Milk Production, Milk Fat Yield, Milk Protein Yield, Energy-Corrected Milk Yield, 3.5% Fat Corrected Milk Yield
- Milk Component Data: Milk Fat Percentage, Milk Protein Percentage, Milk Somatic Cell Count

2.0   STATISTICAL ANALYSIS METHODS

All statistical comparisons of the treatment main effect and two-way interactions with the treatment main effect were performed at the 0.10 level of significance. Statistical analyses were performed using R statistical software version 3.4.0.

Determine the Efficacy of Various Ascus Dairy Rumen Associated Microorganisms
In Live Lactating Dairy Holstein Heifers via Direct Injection into the Rumen

2.1 Fecal ADF and NDF

Fecal ADF (% DM), NDF (% DM), NDFom (% DM), and Dry Matter Percentage values from Study
Days 1, 8, 16, 24, 28 were analyzed using the R package "nlme" and the lme function for linear mixed
models, with treatment, study day, and treatment by study day interaction as fixed effects and Cow ID as
a random effect (where appropriate).

fit <- lme (Response ~ Treatment_Group*Day, random = ~ 1 | ID, data=fecal_data)

Each model was compared to a fixed-effect only model using a Chi-squared test. Least square means
were used to compare treatment groups using the unadjusted p-values and Satterthwaite degrees of
freedom was used to test for significant differences. Tukey's method was used to adjust the p-values for
multiple comparisons.

2.2 Milk Production

The daily total milk production data was transformed into four additional variables: Milk Fat Yield, Milk
Protein Yield, Energy-Corrected Milk Yield, and 3.5% Fat Corrected Milk Yield. Milk Fat Yield was
obtained using the following formula:

$$Milk\ Fat\ Yield = Milk\ Production\ (lbs) \times Milk\ Fat\ Percentage$$

Daily total milk production measurements were transformed into Milk Fat Yield using the average of the
AM and PM Milk Fat Percentages for each cow on the corresponding Study Day. There were no PM Milk
Fat Percentage measurements on Study Days 1 through 7, so the AM measurement was used for the
calculation of Milk Fat Yield on these Study Days instead of the average.

Milk Protein Yield was obtained using the following formula:

$$Milk\ Protein\ Yield = Milk\ Production\ (lbs) \times Milk\ Protein\ Percentage$$

Daily total milk production measurements were transformed into Milk Protein Yield using the average of
the AM and PM Milk Protein Percentages for each cow on the corresponding Study Day. There were no
PM Milk Protein Percentage measurements on Study Days 1 through 7, so the AM measurement was
used for the calculation of Milk Protein Yield on these Study Days instead of the average.

Energy-Corrected Milk Yield was obtained using the following formula:

$$ECM = 0.327 \times Milk\ Production\ (lbs) + 12.95 \times Milk\ Fat\ Yield + 7.2 \times Milk\ Protein\ Yield$$

Daily total milk production measurements were transformed into Energy-Corrected Milk Yield using the
average of the AM and PM Milk Protein Percentages and the average of the AM and PM Milk Fat
Percentages for each cow on the corresponding Study Day. There were no PM Milk Protein Percentage or
Milk Fat Percentage measurements on Study Days 1 through 7, so the AM measurements were used for
the calculation of Energy-Corrected Milk Yield on these Study Days instead of the averages.

Determine the Efficacy of Various Ascus Dairy Rumen Associated Microorganisms
In Live Lactating Dairy Holstein Heifers via Direct Injection into the Rumen

3.5% Fat Corrected Milk Yield was obtained using the following formula:

$$FCM = 0.432 \times Milk\ Production\ (lbs) + 16.23 \times Milk\ Fat\ Yield$$

Daily total milk production measurements were transformed into 3.5% Fat Corrected Milk Yield using
the average of the AM and PM Milk Protein Percentages and the average of the AM and PM Milk Fat
Percentages for each cow on the corresponding Study Day. There were no PM Milk Protein Percentage or
Milk Fat Percentage measurements on Study Days 1 through 7, so the AM measurements were used for
the calculation of 3.5% Fat Corrected Milk Yield on these Study Days instead of the averages.

Milk Production, Milk Fat Yield, Milk Protein Yield, Energy-Corrected Milk Yield, and 3.5% Fat
Corrected Milk Yield measurements from Study Days 1 to 38 were analyzed using the R package "nlme"
and the lme function for linear mixed models, with treatment, week (time period), and the treatment by
week interaction term as fixed effects and Cow ID as a random effect (where appropriate).

> fit <- lme (Response ~ Trt_Group*Time_Period + (1 | Cow_ID), data=avgdataset, na.action =
> na.omit)

Each model was compared to a fixed-effect only model using a Chi-squared test. Least square means
were used to compare treatment groups using the unadjusted p-values and Satterthwaite degrees of
freedom was used to test for significant differences. Tukey's method was used to adjust the p-values for
multiple comparisons.

2.3   Milk Component Data

Milk data (Milk Fat Percentage, Milk Protein Percentage, Milk Somatic Cell Count) measurements from
Study Days 1 to 38 AM and 8 to 38 PM were analyzed using the R package "nlme" and the lme function
for linear mixed models, with treatment, week (time period), and the treatment by week interaction terms
as fixed effects and Cow ID as a random effect (where appropriate). AM and PM measurements were
averaged per study day per cow for analysis. The data for Study Days 1 through 7 were only AM
measurements.

> fit <- lme(Response ~ Trt_Group*Time_Period + (1 | Cow_ID), data=milk_data, na.action =
> na.omit)

Each model was compared to a fixed-effect only model using a Chi-squared test. Least square means
were used to compare treatment groups using the unadjusted p-values and Satterthwaite degrees of
freedom was used to test for significant differences. Tukey's method was used to adjust the p-values for
multiple comparisons. The Milk SCC data were log-transformed.

2.4   Feed Data

The feed data was a set of Dry Matter Percentage, ADF (% DM), NDF (% DM), and NDFom (% DM)
values for samples taken on Study Days 1, 8, 16, 24, and 28. A summary table was produced for this data
set.

Determine the Efficacy of Various Ascus Dairy Rumen Associated Microorganisms
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3.0 RESULTS

Variables are grouped by model outcome (where appropriate): non-significant TRT effect, significant TRT effect, significant TRT*Time Period (week) or TRT*Day effect. Statistically significant results for variables follow, when necessary. Only the appropriate differences are listed and significant differences are denoted with an asterisk (*).

3.1 Fecal ADF and NDF

Fecal ADF (% DM), NDF (% DM), NDFom (% DM), and Dry Matter Percentage were measured from Study Days 1, 8, 16, 24, 28. The R output and code is in Appendix A.

Table 3.1.1 Model Information for Fecal Data

Variable	Model Type	P-values		Decision
		Treatment_Group	Treatment_Group*Study_Day	
ADF (% DM)	Fixed Effects Only	0.2433	0.1497	(1)
NDF (% DM)	Fixed Effects Only	0.2833	0.05478	(3)
NDFom (% DM)	Fixed Effects Only	0.2386	0.1796	(1)
Dry Matter %	Fixed Effects Only	0.03432	0.1777	(2)

Decisions:

(1) There were no significant terms involving Treatment_Group. No further evaluation is needed.

(2) The Treatment_Group main effect is significant at $\alpha=0.10$, and the 2-way interaction is not significant. Compare treatment means from the main effect of Treatment_Group.

(3) The Treatment_Group by Study_Day interaction is significant at $\alpha=0.10$. Compare treatment means within each day.

Table 3.1.2 Decision (2) Fecal Data Dry Matter %: Differences and Standard Deviations for Treatment Effects

Variable	Compare	Difference	Standard Error	P-value	Significance
Dry Matter %	Treatment Group 1 vs. Treatment Group 2	0.005060	0.005165	0.5914	
Dry Matter %	Treatment Group 1 vs. Treatment Group 3	0.01416	0.005272	0.0229	*
Dry Matter	Treatment Group 2 vs. Treatment Group 3	0.009096	0.005201	0.1923	

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%						

For Dry Matter Percentage, Treatment Group 1 had significantly higher values than Treatment Group 3, p=0.0229.

For decision (3) for NDF (% DM), only the significant contrasts between treatments within Study Days are displayed. The R output and code is in Appendix A.

Table 3.1.3 Decision (3) Fecal Data NDF (% DM): Significant Differences and Standard Deviations for Treatment Effects

Variable	Study Day	Compare	Difference	Standard Error	P-value	Significance
NDF (% DM)	1	Treatment Group 1 vs. Treatment Group 2	0.1081	0.03793	0.0146	*
NDF (% DM)	1	Treatment Group 2 vs. Treatment Group 3	-0.08360	0.03665	0.0631	*

For NDF (% DM), Treatment Group 1 had significantly higher values than Treatment Group 2 on Day 1, p=0.0146. Treatment Group 2 had significantly lower values than Treatment Group 3 on Day 1, p=0.0631.

3.2   Milk Production

Daily total milk production (sum of AM and PM) measurements were taken on Study Days -7 to 38, but only the measurements from Study Days 1 to 38 were analyzed. Descriptions of the calculations performed to obtain all variables in this section are given in Section 2.2. The R output and code is in Appendix A.

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| Table 3.2.1: Model Information for Milk Production Data				
Variable	Model Type	P-values		Decision
		Treatment_Group	Treatment_Group*Week	
Milk Production	Mixed	0.3233	<0.0001	(3)
Milk Fat Yield	Mixed	0.637	0.022	(3)
Milk Protein Yield	Mixed	0.5017	<0.0001	(3)
Energy-Corrected Milk Yield	Mixed	0.4284	<0.0001	(3)
3.5% Fat-Corrected Milk Yield	Mixed	0.4348	<0.0001	(3)

Decisions:

(1) There were no significant terms involving Treatment_Group. No further evaluation is needed.

(2) The Treatment_Group main effect is significant at $\alpha=0.10$, and the 2-way interaction is not significant. Compare treatment means from the main effect of Treatment_Group.

(3) The Treatment_Group by Week interaction is significant at $\alpha=0.10$. Compare treatment means within each week.

Table 3.2.2: Decision (3) Milk Production Data Differences and Standard Deviations for Treatment Effects					
Variable	Week	Compare	Difference	Standard Error	P-value
Milk Production	2	Treatment Group 2 vs. Treatment Group 3	17.3214	5.7712	0.0185
Milk Production	3	Treatment Group 2 vs. Treatment Group 3	13.4107	5.7712	0.0754
Milk Protein Yield	2	Treatment Group 2 vs Treatment Group 3	0.5191	0.1872	0.0302
Energy-Corrected Milk Yield	2	Treatment Group 1 vs Treatment Group 3	13.1688	5.9642	0.0942
Energy-Corrected Milk Yield	2	Treatment Group 2 vs Treatment Group 3	15.9651	5.7620	0.0303
3.5% Fat-Corrected Milk Yield	2	Treatment Group 2 vs Treatment Group 3	15.7085	5.9674	0.0405

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For Milk Production, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0185. Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 3, p=0.0754.

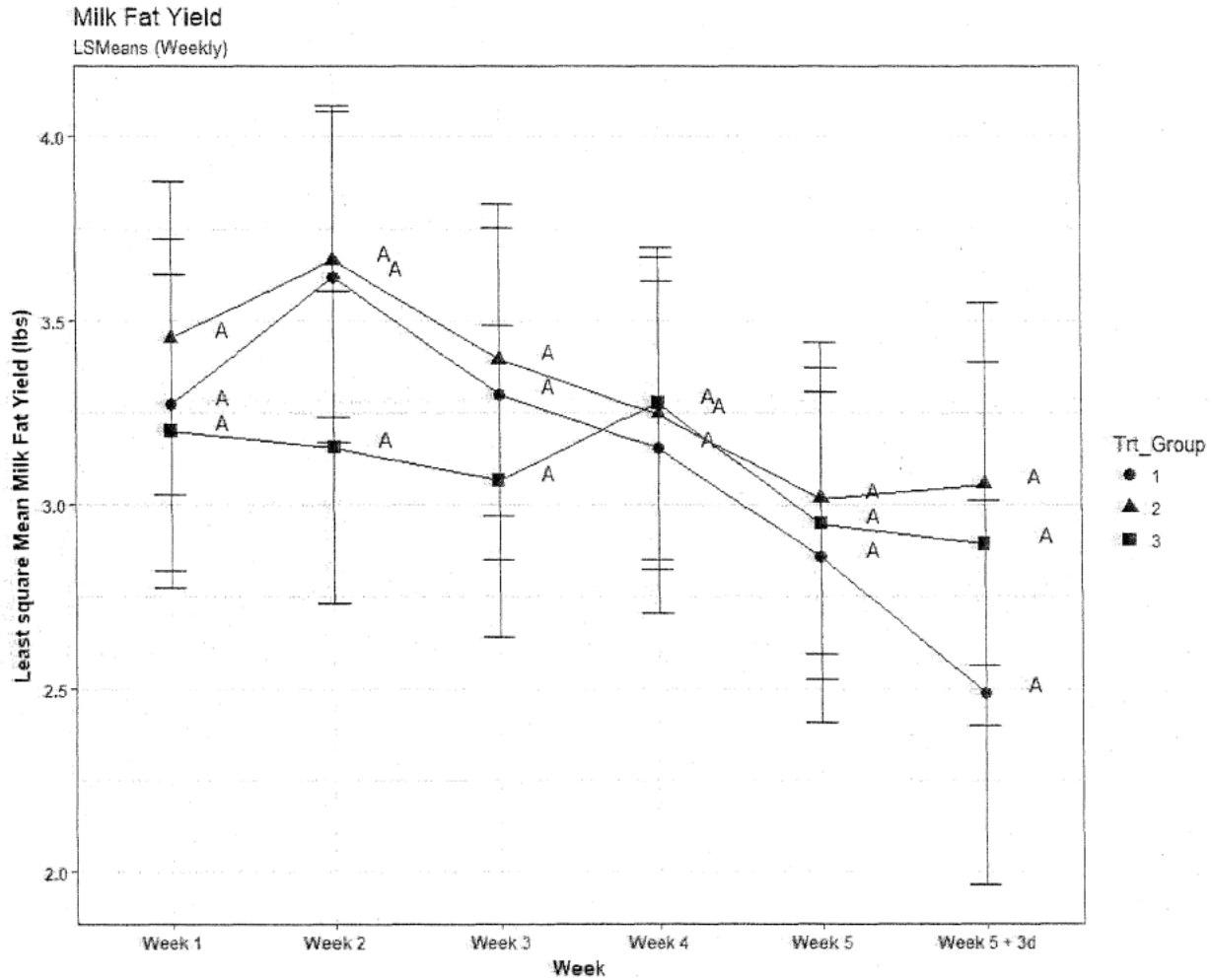Figure 3.2.1: Graph of Weekly Least Square Means for Milk Production
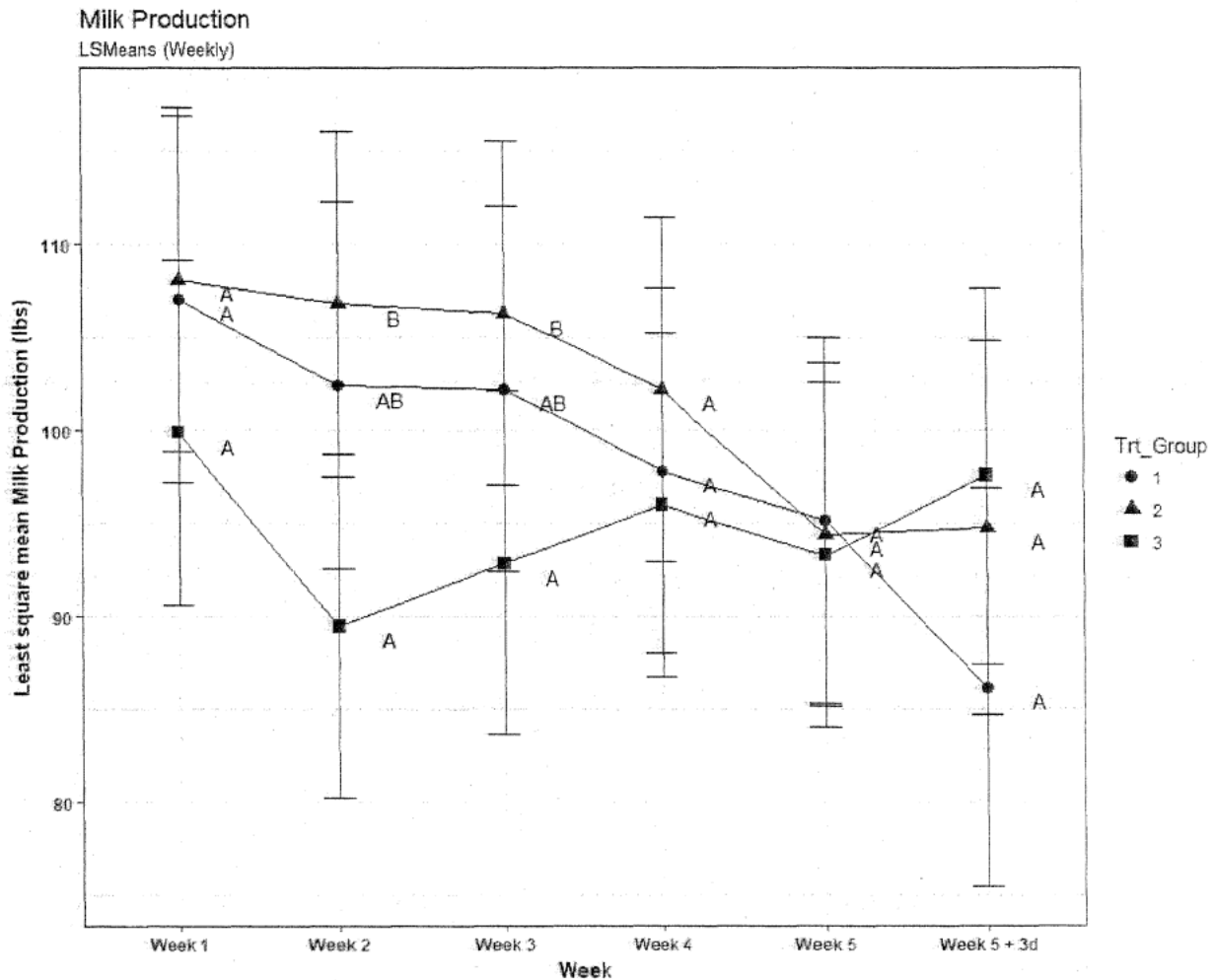
Milk Production (daily total, lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Determine the Efficacy of Various Ascus Dairy Rumen Associated Microorganisms
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Although the Treatment Group by Week interaction was significant, there were no significant individual Treatment Group LSMean differences within week for Milk Fat Yield. The adjustment for multiple comparisons created this disparity.

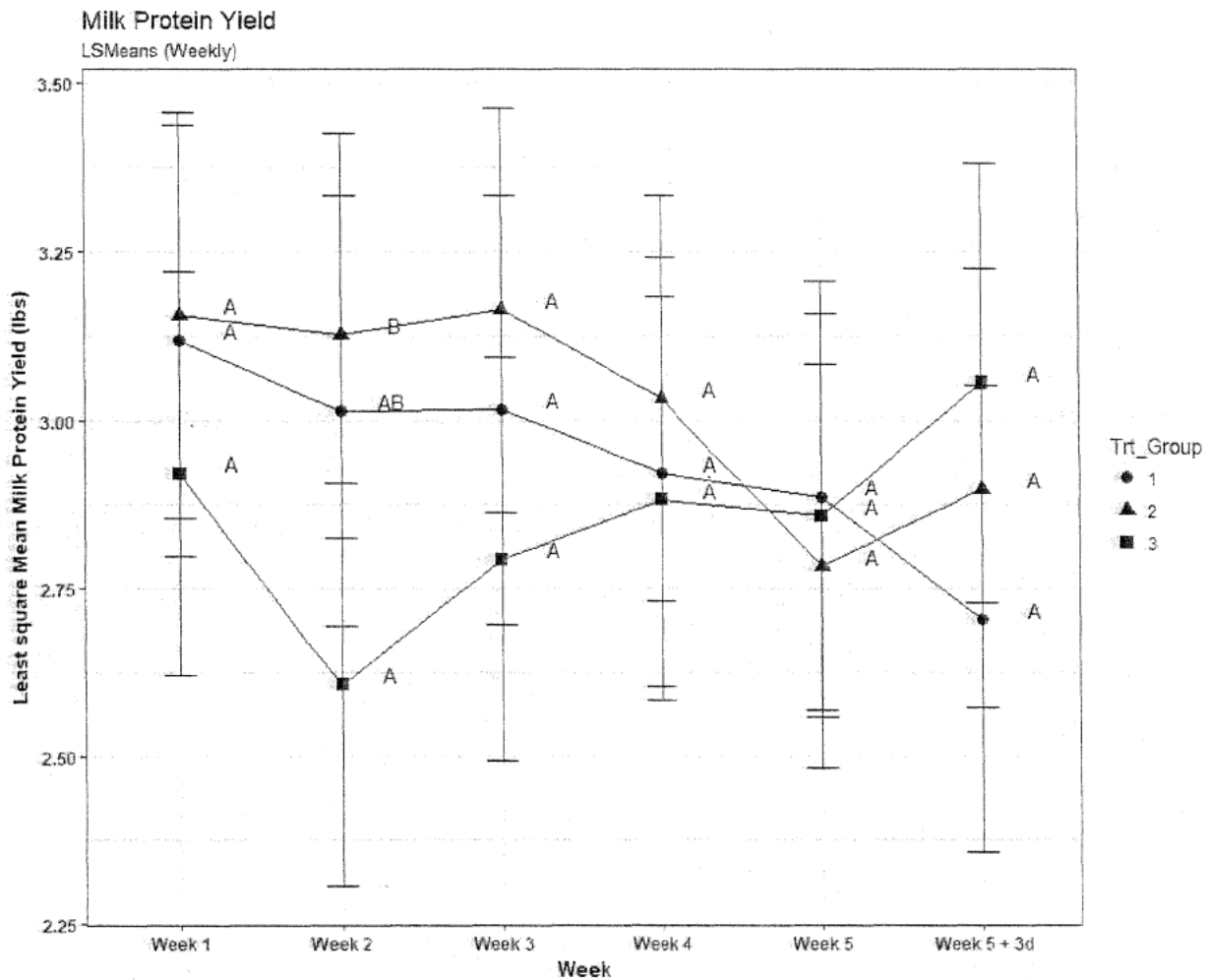Figure 3.2.2: Graph of Weekly Least Square Means for Milk Fat Yield

Milk Fat Yield (lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

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For Milk Protein Yield, Treatment Group 2 had significantly higher values than Treatment Group 3
during Week 2, p=0.0302.

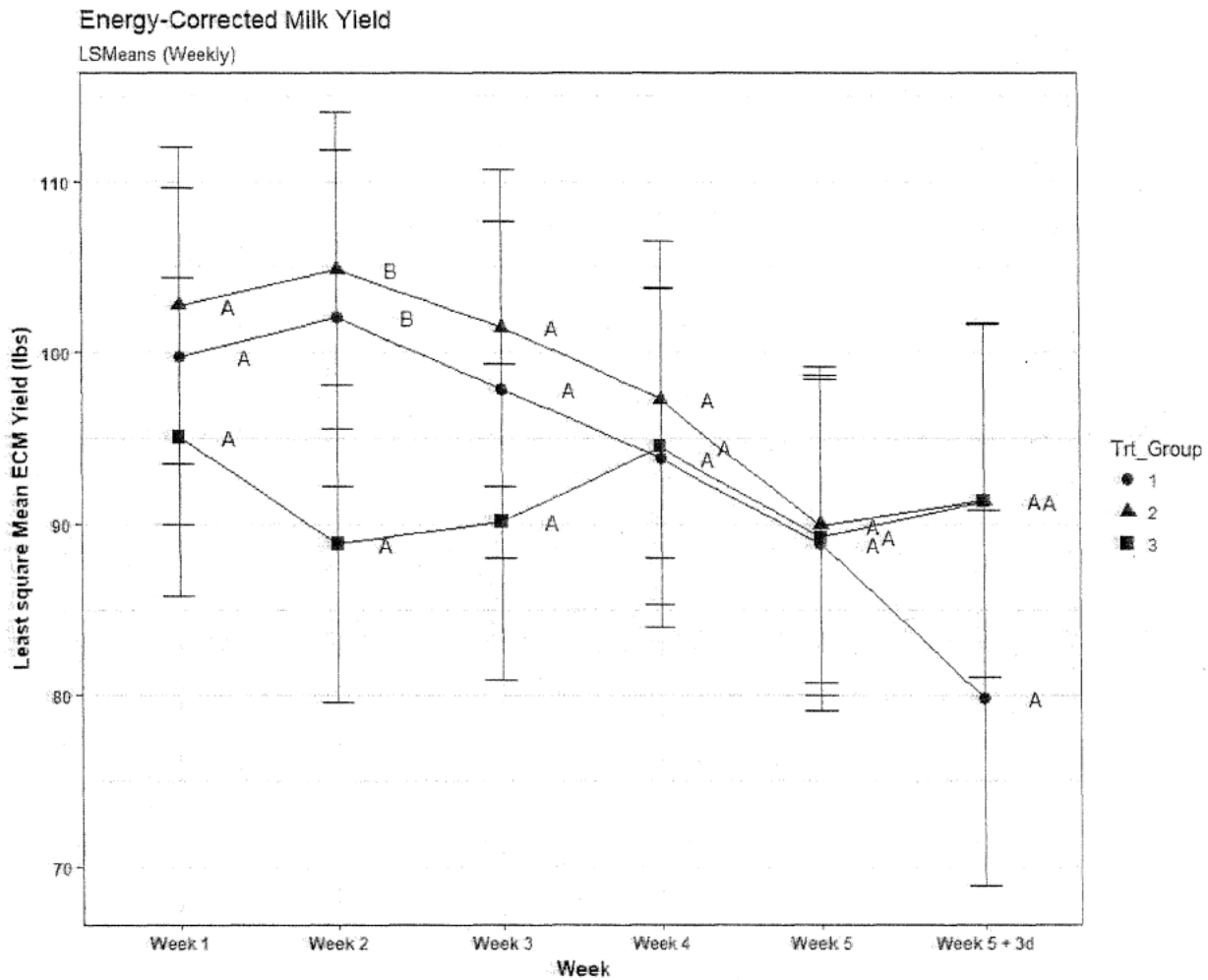Figure 3.2.3: Graph of Weekly Least Square Means for Milk Protein Yield

Milk Protein Yield (lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars
indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted
comparisons).

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For Energy-corrected Milk Yield, Treatment Group 1 had significantly higher values than Treatment Group 3 during Week 2, p=0.0942. Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 2, p=0.0303.

Figure 3.2.4: Graph of Weekly Least Square Means for Energy-Corrected Milk Yield

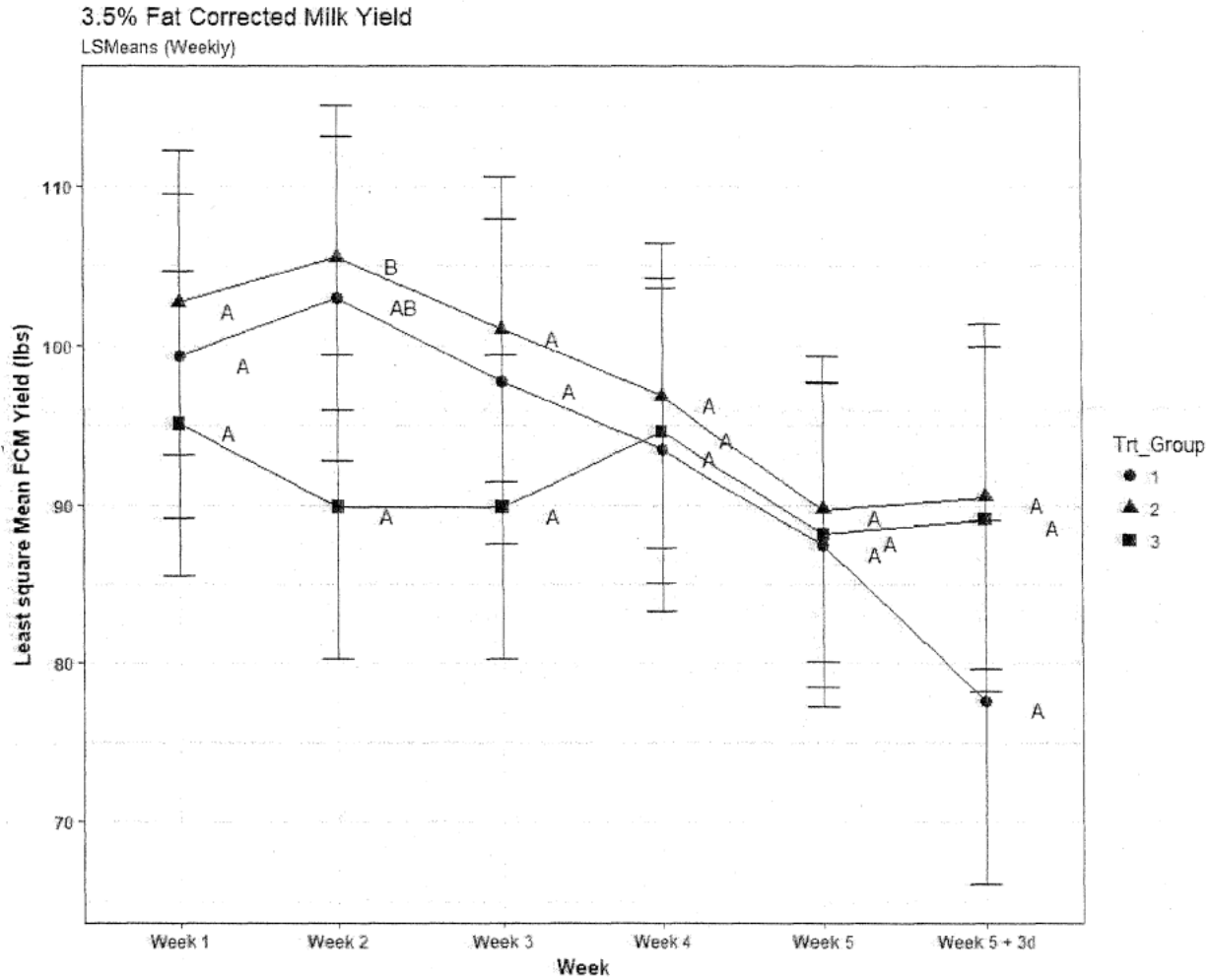**Energy-Corrected Milk Yield**

Energy-Corrected Milk Yield (lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

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For 3.5% Fat-Corrected Milk Yield, Treatment Group 2 had significantly higher values than Treatment
Group 3 during Week 2, p=0.0405.

Figure 3.2.5: Graph of Weekly Least Square Means for 3.5% Fat Corrected Milk Yield

3.5% Fat Corrected Milk Yield (lbs) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars
indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted
comparisons).

3.3   Milk Component Data

Milk data (Milk Fat Percentage, Milk Protein Percentage, Milk Lactose Percentage, Milk Solids
Percentage, Milk Somatic Cell Count) were measured on Study Days -7 to 38 for AM measurements, and
on Study Days 8 to 38 for PM measurements. The milk data from Study Days 1 to 38 were analyzed. AM
and PM measurements were averaged per study day per cow for analysis. The data for Study Days 1
through 7 were only AM measurements. The R output and code is in Appendix A.

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Table 3.3.1: Model Information for Milk Component Data				Decision
Variable	Model Type	P-values		
		Treatment_Group	Treatment_Group*Week	
Milk Fat %	Mixed	0.8392	0.1733	(1)
Milk Protein %	Mixed	0.7404	<0.0001	(3)
Milk SCC	Mixed	0.1310	0.0218	(3)

Decisions:
(1) There were no significant terms involving Treatment_Group. No further evaluation is needed.
(2) The Treatment_Group main effect is significant at α=0.10, and the 2-way interaction is not significant. Compare treatment means from the main effect of Treatment_Group.
(3) The Treatment_Group by Week interaction is significant at α=0.10. Compare treatment means within each week.

Table 3.3.2: Decision (3) Differences and Standard Deviations for Treatment Effects					
Variable	Week	Compare	Difference	Standard Error	P-value
Milk Protein %	5 + 2d	Treatment Group 1 vs. Treatment Group 2	0.5731	0.1051	0.0001
Milk Protein %	5 + 2d	Treatment Group 1 vs. Treatment Group 3	0.4569	0.1051	0.0009
Milk SCC	1	Treatment Group 2 vs Treatment Group 3	1.494	0.5299	0.0273

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There were no significant individual Treatment Group LSMean differences within week for Milk Fat %.

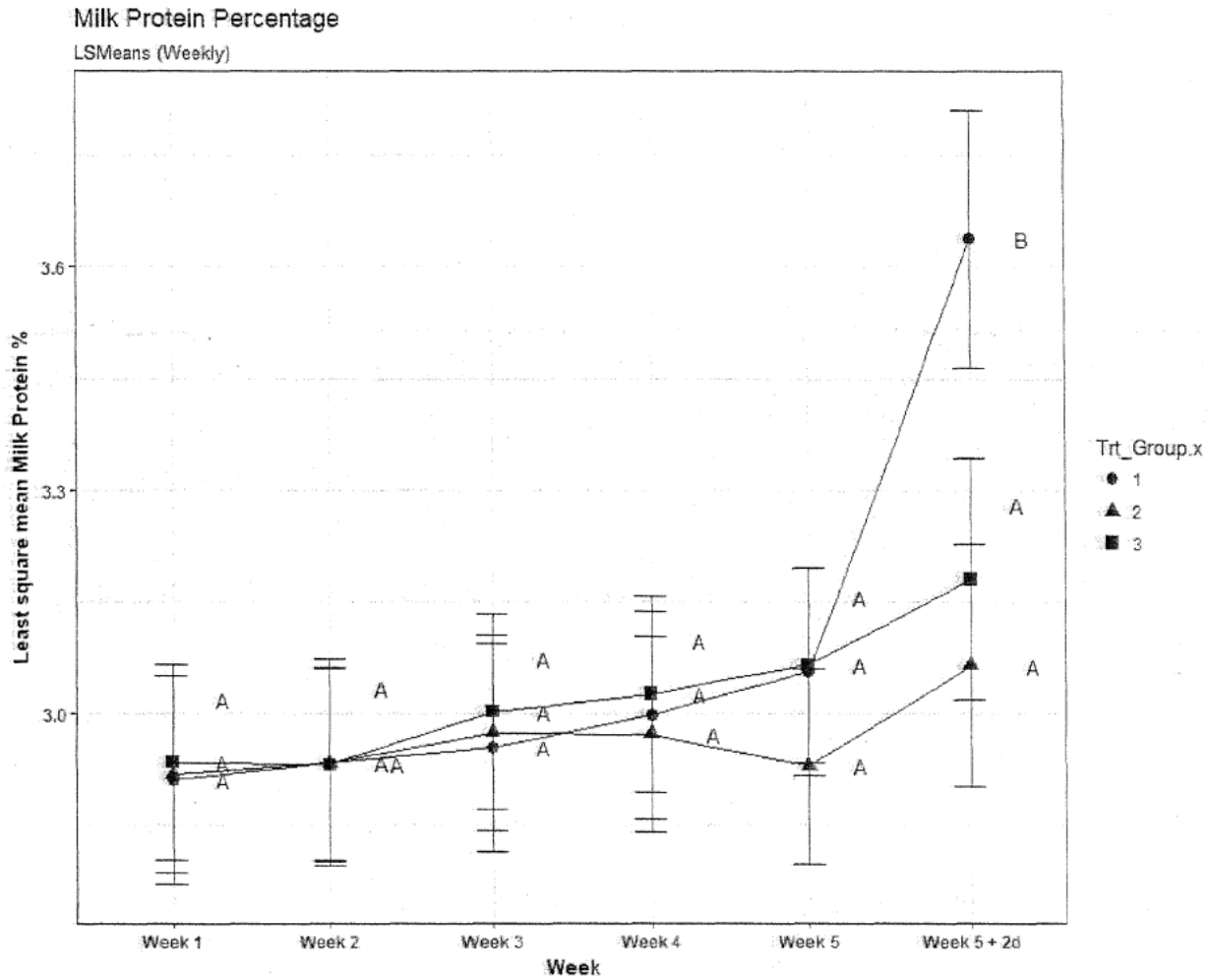Figure 3.3.1: Graph of Weekly Least Square Means for Milk Fat Percentage
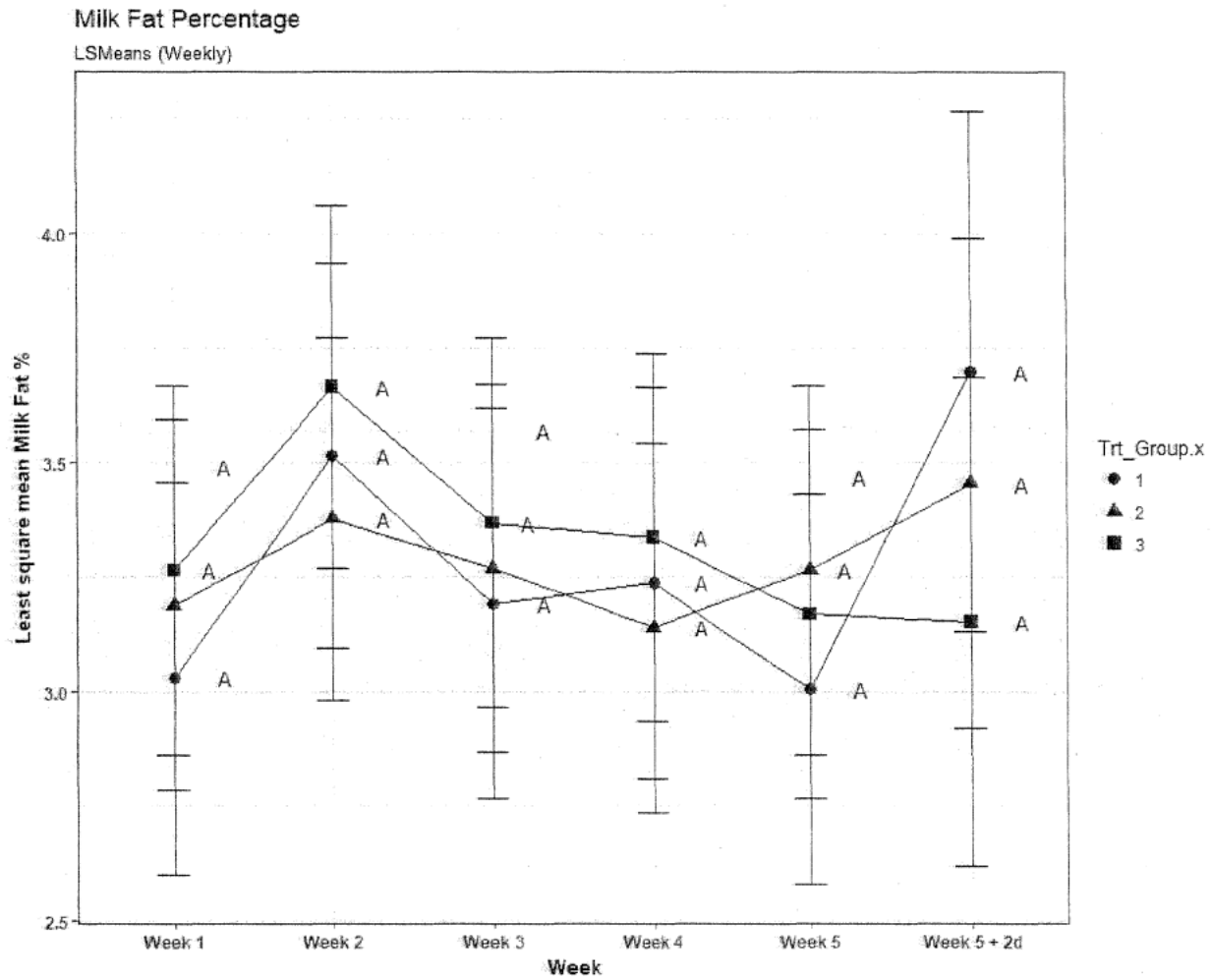
Milk Fat Percentage for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars
indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted
comparisons).

Determine the Efficacy of Various Ascus Dairy Rumen Associated Microorganisms
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For Milk Protein %, Treatment Group 1 had significantly higher values than Treatment Group 2 during Week 5 + 2d, p=0.0001. Treatment Group 1 had significantly higher values than Treatment Group 3 during Week 5 + 2d, p=0.0009.

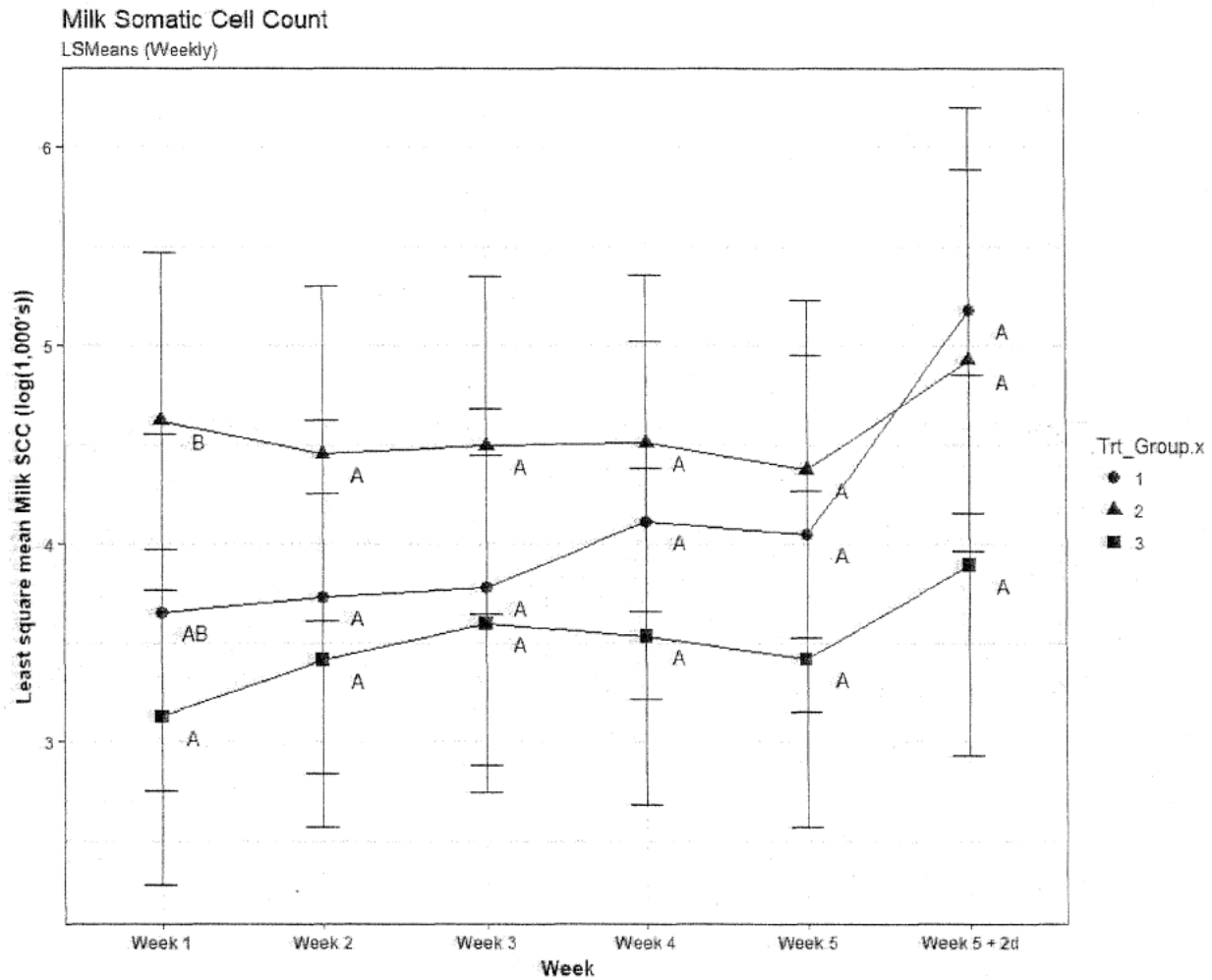Figure 3.3.2: Graph of Weekly Least Square Means for Milk Protein Percentage

Milk Protein Percentage for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

Determine the Efficacy of Various Ascus Dairy Rumen Associated Microorganisms
In Live Lactating Dairy Holstein Heifers via Direct Injection into the Rumen

For Milk SCC, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week 1, p=0.0273.

Figure 3.3.3: Graph of Weekly Least Square Means for Milk Somatic Cell Count

Milk Somatic Cell Count (log(1,000's)) for 3 treatment groups over 5 weeks. Shapes indicate the weekly LS means. Error bars indicate the 90% confidence interval of the LS mean. Means sharing a letter are not significantly different (Tukey-adjusted comparisons).

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3.4   Feed Data

For feed, Dry Matter Percentage, ADF (% DM), NDF (% DM), and NDFom (% DM) values for samples
were measured on Study Days 1, 8, 16, 24, and 28.

Table 3.4.1 Summary of Feed Data	Dry Matter Percentage	ADF (% DM)	NDF (% DM)	NDFom (% DM)
Minimum	0.4654	0.1901	0.2764	0.2587
1st Quartile	0.4755	0.1978	0.2835	0.2636
Median	0.4847	0.1985	0.2879	0.2649
Mean	0.4813	0.1982	0.2861	0.2648
3rd Quartile	0.4855	0.1998	0.2892	0.2667
Maximum	0.4952	0.2046	0.2937	0.2702
Standard Deviation	0.01128	0.005229	0.006547	0.004226
Coefficient of Variation (%)	2.344	2.639	2.288	1.596

4.0   CONCLUSIONS

Statistically significant differences between treatment groups were determined to be as follows:

For Fecal Data Dry Matter Percentage, Treatment Group 1 had significantly higher values than Treatment
Group 3, p=0.0229.

For Fecal Data NDF (% DM), Treatment Group 1 had significantly higher values than Treatment Group 2
on Day 1, p=0.0146. Treatment Group 2 had significantly lower values than Treatment Group 3 on Day 1,
p=0.0631.

For Milk Protein %, Treatment Group 1 had significantly higher values than Treatment Group 2 during
Week 5 + 2d, p=0.0001. Treatment Group 1 had significantly higher values than Treatment Group 3
during Week 5 + 2d, p=0.0009.

For Milk SCC, Treatment Group 2 had significantly higher values than Treatment Group 3 during Week
1, p=0.0273.

For Milk Production, Treatment Group 2 had significantly higher values than Treatment Group 3 during
Week 2, p=0.0185. Treatment Group 2 had significantly higher values than Treatment Group 3 during
Week 3, p=0.0754.

For Milk Fat Yield, Treatment Group 1 had significantly higher values than Treatment Group 2 during
Week 1, p=0.0905.

For Milk Protein Yield, Treatment Group 1 had significantly higher values than Treatment Group 2
during Week 1, p=0.0251. Treatment Group 2 had significantly lower values than Treatment Group 3
during Week 1, p=0.0932.

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For Energy-corrected Milk Yield, Treatment Group 2 had significantly higher values than Treatment
Group 3 during Week 2, p=0.0614.

For 3.5% Fat-Corrected Milk Yield, Treatment Group 2 had significantly higher values than Treatment
Group 3 during Week 2, p=0.0405.

Cerrito, Chelsea

From:	Kristi Smedley <smedley@cfr-services.com>
Sent:	Friday, December 17, 2021 11:04 AM
To:	Cerrito, Chelsea
Cc:	Animalfood-premarket; Kevin Korth
Subject:	RE: [EXTERNAL] RE: GRAS Notice No. AGRN 41 - Call regarding Clostridium beijerinckii ASCUSDY20 for use as a viable microorganism in diets of dairy cattle
Attachments:	CFR DRAFT LETTER to CEASE REVIEW AGRN 41-December 17 2021.pdf

> CAUTION: This email originated from outside of the organization. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Ms. Cerrito:

I have attached the request for cease evaluating AGRN 41.

Please let me know if you have any issues in downloading the letter.

**RECEIVED DATE
DEC 20, 2021**

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell _____ (b) (6)
Fax 703-580-8637

From: Cerrito, Chelsea [mailto:Chelsea.Cerrito@fda.hhs.gov]
Sent: Friday, December 03, 2021 2:22 PM
To: Kristi Smedley
Subject: RE: [EXTERNAL] RE: GRAS Notice No. AGRN 41 - Call regarding Clostridium beijerinckii ASCUSDY20 for use as a viable microorganism in diets of dairy cattle

Dear Kristi,

Please find attached our response letter and November 12, 2021 meeting minutes (both dates December 3, 2021).

Kind regards,
Chelsea

From: Kristi Smedley <smedley@cfr-services.com>
Sent: Monday, November 15, 2021 10:40 AM
To: Cerrito, Chelsea <Chelsea.Cerrito@fda.hhs.gov>
Subject: [EXTERNAL] RE: GRAS Notice No. AGRN 41 - Call regarding Clostridium beijerinckii ASCUSDY20 for use as a viable microorganism in diets of dairy cattle

As noted in the meeting, we are requesting note do the Friday, November 12, meeting.   We will accept electronically.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell   (b) (6)
Fax 703-580-8637

From: Cerrito, Chelsea [mailto:Chelsea.Cerrito@fda.hhs.gov]
Sent: Thursday, November 04, 2021 7:34 AM
To: Kristi Smedley (smedley@cfr-services.com)
Subject: GRAS Notice No. AGRN 41 - Call regarding Clostridium beijerinckii ASCUSDY20 for use as a viable microorganism in diets of dairy cattle

Dear Dr. Smedley,

We would like to schedule a call with you and representatives from Native Microbials, Inc. to discuss AGRN 41 regarding *Clostridium beijerinckii* ASCUSDY20 for use as a viable microorganism in diets of dairy cattle. The purpose of the call is to provide an update on the evaluation of the GRAS notice.

We are available for a one hour call on Friday, November 12 from 11am – 12pm, 12 – 1pm, or 2 – 3pm US Eastern Time. We are also available on Monday, November 15 from 3 – 4pm US Eastern Time. Please let me know if one of these times works for your client.
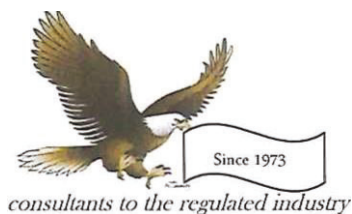
Kind Regards,
Chelsea

Chelsea Cerrito, MAS
Animal Scientist, Division of Animal Feeds (DAF)

Center for Veterinary Medicine
Office of Surveillance and Compliance
U.S. Food and Drug Administration
Tel: 240-402-6729
Personal e-mail address: Chelsea.Cerrito@fda.hhs.gov
To schedule a meeting with DAF, please e-mail: animalfood-premarket@fda.hhs.gov

Center for Regulatory Services, Inc.

5200 Wolf Run Shoals Road
Woodbridge, VA 22192-575.5
703 *590* 7337 (Fax 703 580 8637)
Smedley@cfr-services.com

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December 17, 2021

David Edwards Director

Division of Animal Feeds (HFV- 220)

Center for Veterinary Medicine

Food and Drug Administration

7519 Standish Pl.

Rockville, MD 20855

> Subject: Animal GRAS Notice #41
>
> DFM *Clostridium beijerinckii* ASCUSDY20
>
> Notifier: Native Microbials, Inc.
>
> 10255 Science Center Dr. Suite C2
>
> San Diego, California 92121

Dear Dr. Edwards:

On behalf of Native Microbials, Inc. (previously known as ASCUS BioSciences, Inc.), I am requesting that you cease evaluating their animal GRAS notice 41 specific to *Clostridium beijerinckii* ASCUSDY20.*

Should you have any questions on the filing, please contact me directly.

Sincerely,

Kristi
Smedley

Digitally signed by Kristi Smedley
DN: cn=Kristi Smedley, o=Center
for Regulatory Services, Inc., ou,
email=smedley@cfr-
services.com, c=US
Date: 2021.12.17 10:53:48 -05'00'

Kristi O. Smedley, Ph.D.
Consultant to Native Microbials, Inc.

Cc: Mallory Embree, Native Microbials, Inc.